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(54) Title: REAGENTS BINDING VINCULIN, DYNEIN, AND GLUTATHIONE S-TRANSFERASE FROM PEPTIDE LIBRARIES

(57) Abstract

A novel method for producing novel and/or improved heterofunctional binding fusion proteins termed Totally Synthetic Affinity Reagents (TSARs) that have affinity for the ligands vinculin, dynein, or glutathione S-transferase is disclosed. Novel and/or improved heterofunctional binding reagents to vinculin, dynein, or glutathione S-transferase as well as methods for using the reagents for a variety of *in vitro* and *in vivo* applications are also disclosed. Also disclosed are methods for identifying inhibitors of enzymes by the use of random peptide libraries.

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REAGENTS BINDING VINCULIN, DYNEIN, AND GLUTATHIONE S-TRANSFERASE FROM PEPTIDE LIBRARIES

This application is a continuation-in-part of co-pending application Serial No. 08/189,331 filed January 31, 1994 the entire disclosure of which is incorporated herein by reference.

1. FIELD OF THE INVENTION

The present invention relates generally to methods of screening peptide libraries for peptides having binding specificity for vinculin, dynein, or glutathione S-transferase. The invention further relates to novel peptides identified according to the methods of the invention as well as compositions comprising the binding domains of such peptides or a portion thereof having the same binding specificity. Also provided is a method for identifying inhibitors of enzymes.

2. BACKGROUND OF THE INVENTION

2.1. PEPTIDE LIBRARIES

There have been two different approaches to the construction of random peptide libraries. According to one approach, peptides have been chemically synthesized in vitro in several formats. Examples of chemically synthesized libraries can be found in Fodor, S., et al., 1991, Science 251: 767-773; Houghten, R., et al., 1991, Nature 354: 84-86; and Lam, K., et al., 1991, Nature 354: 82-84.

A second approach to the construction of random peptide libraries has been to use the M13 phage, and, in particular, protein pIII of M13. The viral capsid protein of M13, protein III (pIII), is responsible for infection of bacteria. Several investigators have determined from mutational analysis that the 406 amino acid long pIII capsid protein has two domains. The C-terminus anchors the protein to the viral coat, while portions of the N-terminus of pIII are essential for interaction with the E. coli pillin protein

(Crissman, J.W. and Smith, G.P., 1984, Virology 132: 445-455). Although the N-terminus of the pIII protein has shown to be necessary for viral infection, the extreme N-terminus of the mature protein does tolerate alterations. In 1985, George Smith published experiments reporting the use of the pIII protein of bacteriophage M13 as an experimental system for expressing a heterologous protein on the viral coat surface (Smith, G.P., 1985, Science 228: 1315-1317). It was later recognized, independently by two groups, that the M13 phage pIII gene display system could be a useful one for mapping antibody epitopes (De la Cruz, V., et al., 1988, J. Biol. Chem. 263: 4318-4322; Parmley, S.F. and Smith, G.P., 1988, Gene 73: 305-318).

Parmley, S.F. and Smith, G.P., 1989, Adv. Exp. Med. Biol. 251: 215-218 suggested that short, synthetic DNA segments cloned into the pIII gene might represent a library of epitopes. These authors reasoned that since linear epitopes were often ~6 amino acids in length, it should be possible to use a random recombinant DNA library to express all possible hexapeptides to isolate epitopes that bind to antibodies. Scott, J.K. and Smith, G.P., 1990, Science 249: 386-390 describe construction and expression of an "epitope library" of hexapeptides on the surface of M13. Cwirla, S.E., et al., 1990, Proc. Natl. Acad. Sci. USA 87: 6378-6382 also described a somewhat similar library of hexapeptides expressed as gene pIII fusions of M13 fd phage. PCT Application WO 91/19818 published December 26, 1991 by Dower and Cwirla describes a similar library of pentameric to octameric random amino acid sequences. Devlin et al., 1990, Science, 249: 404-406, describes a peptide library of about 15 residues generated using an (NNS) coding scheme for oligonucleotide synthesis in which S is G or C. Christian and colleagues have described a phage display library, expressing decapeptides (Christian, R.B., et al., 1992, J. Mol. Biol. 227: 711-718).

Other investigators have used other viral capsid proteins for expression of non-viral DNA on the surface of phage particles. For example,

the major capsid protein pVIII was so used by Cesareni, G., 1992, FEBS Lett. 307: 66-70. Other bacteriophage than M13 have been used to construct peptide libraries. Four and six amino acid sequences corresponding to different segments of the Plasmodium falciparum major surface antigen have been cloned and expressed in the filamentous bacteriophage fd (Greenwood, J., et al., 1991, J. Mol. Biol. 220: 821-827).

Kay et al., 1993, Gene 128: 59-65 (Kay) discloses a method of constructing peptide libraries that encode peptides of totally random sequence that are longer than those of any prior conventional libraries. The libraries disclosed in Kay encode totally synthetic random peptides of greater than about 20 amino acids in length. Such libraries can be advantageously screened to identify peptides, polypeptides and/or proteins having binding specificity for a variety of ligands.

2.2. VINCULIN

Vinculin is a 130 kD protein found in all cells. In cardiac muscle it is found in intercalated disks and in smooth muscle it is found in membrane associated plaques. It is involved in the junctions and adhesion belts which join epithelial cells into sheets at cell-to-cell junctions.

Vinculin is found in focal adhesions which are sites of interaction between the cytoskeleton and the extracellular matrix. Vinculin interacts with a number of other proteins that are also present in focal adhesions including talin, α-actinin, paxilln, and actin. Vinculin is involved in the structure of tissues, cell movement, wound healing, tumor metastasis, development, and the process of the regulation of cell growth and division.

2.3. DYNEIN

Dynein is a large enzyme complex that is involved in intracellular movement associated with microtubules. Dynein is an integral part of the process by which chemical energy in the form of adenosine

triphosphate (ATP) is converted into mechanical energy. The dynein complex is made up of proteins having a molecular weight of between 1000 and 2000 kD. In cilia and flagella of microorganisms, dynein drives the generation of force in the axonemal structure. In higher cells, dynein has been shown to be involved in the poleward migration of chromosomes during mitosis, sorting between the apical and bosolateral surfaces of epithelial cells, and retrograde axonal transport, among other processes. See Vallee, 1993, Proc. Natl. Acad. Sci. USA 90: 8769-8772.

2.4. GLUTATHIONE S-TRANSFERASE

Glutathione S-transferase (GST) is a dimeric enzyme that conjugates glutathione (GSH) to various other substrates, including products of tissue damage and carcinogens (Gilliand, G.L., 1993, Current Opinion in Structural Biology 3: 875-884; Pickett, C.B. and Lu, A.Y.H., 1989, Annual Reviews Biochemistry 58: 743-764). Its role in the cell seems to be in detoxification. It has been shown that in some tumor cells resistant to anti-cancer drugs the levels of GST or GST activity are elevated (Pickett, C.B. and Lu, A.Y.H., 1989, Annual Reviews Biochemistry 58: 743-764; Tew, K.D., 1994, Cancer Res. 54: 4313-4320). GST has also become known extensively by molecular biologists due to its use in the E. coli expression system pGEX (Smith, D.B. and Johnson, K.S., 1988, Gene 67: 31-40). pGEX directs the expression of foreign polypeptides as fusions to GST. These fusion proteins can be easily purified through chromatography and elution due to the affinity of GST for GSH.

Citation or identification of any reference in Section 2 of this application shall not be construed as an admission that such reference is available as prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention provides methods for identifying proteins/polypeptides and/or peptides that bind specifically to vinculin, dynein, or glutathione S-transferase. Such proteins/polypeptides and/or peptides can be identified using any random peptide library, *e.g.*, a chemically synthesized peptide library or a biologically expressed peptide library. If a biological peptide expression library is used, the nucleic acid which encodes the peptide which binds to the ligand of choice can be recovered, and then sequenced to determine its nucleotide sequence and hence deduce the amino acid sequence that mediates binding. Alternatively, the amino acid sequence of an appropriate binding domain can be determined by direct determination of the amino acid sequence of a peptide selected from a peptide library containing chemically synthesized peptides. In a less preferred aspect, direct amino acid sequencing of a binding peptide selected from a biological peptide expression library can also be performed.

In a particular embodiment, such proteins/polypeptides and/or peptides are called Totally Synthetic Affinity Reagents (TSARs). As used in the present invention, a TSAR is intended to encompass a concatenated heterofunctional protein, polypeptide and/or peptide that includes at least two distinct functional regions. One region of the heterofunctional TSAR is a binding domain with affinity for a ligand that is preferably characterized by 1) its strength of binding under specific conditions, 2) the stability of its binding under specific conditions, and 3) its selective specificity for the chosen ligand. A second region of the heterofunctional TSAR is an effector domain that is biologically or chemically active to enhance expression and/or detection and/or purification of the TSAR. In a preferred aspect, the TSAR is a fusion protein in which the effector domain is at least a functional portion of a phage structural protein (*e.g.*, pIII).

A TSAR can contain an optional additional linker domain or region between the binding domain and the effector domain. The linker region serves (1) as a structural spacer region between the binding and effector domains; (2) as an aid to uncouple or separate the binding and effector domains; or (3) as a structural aid for display of the binding domain and/or the TSAR by the expression vector.

The present invention further provides novel reagents as well as compositions comprising a binding domain of a protein/polypeptide and/or peptide or of a TSAR or a portion thereof having specificity for a ligand selected from among vinculin, dynein, or enzymes such as glutathione S-transferase. Also provided are methods for identifying a protein/polypeptide and/or peptide which inhibits the activity of an enzyme, *e.g.*, glutathione S-transferase. Where the ligand of choice possesses enzymatic activity, the present invention provides reagents as well as compositions that are useful as inhibitors of the enzymatic activity of the ligand.

The present invention further provides reagents and compositions that bind to vinculin. Such reagents and compositions can be used in cell culture, for the affinity purification of vinculin, as histological reagents, for altering the mobility or attachability of malignant cells, as well as modulating platelet release and blood clotting. Also provided are methods of identifying a series of reagents and compositions that show progressively increased binding affinity for vinculin.

A library of recombinant vectors may be generated or constructed to express a plurality of heterofunctional fusion proteins, polypeptides and/or peptide TSARs. In a preferred embodiment, the methods utilize TSARs that are expressed on the surface of the recombinant vectors of the library.

In a particular embodiment, the present invention encompasses a method for identifying a protein, polypeptide and/or peptide which binds to a ligand selected from among vinculin, dynein, and glutathione S-transferase

comprising: screening, for example, a library of recombinant vectors which express a plurality of heterofunctional fusion proteins comprising (a) a binding domain encoded by an oligonucleotide comprising unpredictable nucleotides in which the unpredictable nucleotides are arranged in one or more contiguous sequences, wherein the total number of unpredictable nucleotides is greater than or equal to 60 and less than or equal to 600, and (b) an effector domain encoded by an oligonucleotide sequence which is a protein or peptide that enhances expression or detection of the binding domain, by contacting the plurality of heterofunctional fusion proteins with the ligand under conditions conducive to ligand binding and isolating the fusion proteins which bind to the ligand. Alternatively, the present invention encompasses a method for identifying a protein and/or peptide which binds to a ligand selected from among vinculin, dynein, and glutathione S-transferase comprising: (a) generating a library of vectors expressing a plurality of heterofunctional fusion proteins comprising (i) a binding domain encoded by a double stranded oligonucleotide comprising unpredictable nucleotides in which the unpredictable nucleotides are arranged in one or more contiguous sequences, wherein the total number of unpredictable nucleotides is greater than or equal to 60 and less than or equal to 600, and (ii) an effector domain encoded by an oligonucleotide sequence encoding a protein or peptide that enhances expression or detection of the binding domain; and (b) screening the library of vectors by contacting the plurality of heterofunctional fusion proteins with the ligand under conditions conducive to ligand binding and isolating the heterofunctional fusion protein which binds to the ligand. Additionally, the methods of the invention further comprise determining the nucleotide sequence encoding the binding domain of the heterofunctional fusion protein identified to deduce the amino acid sequence of the binding domain.

In order to prepare a library of recombinant vectors expressing a plurality of protein, polypeptide and/or peptide TSARs, single stranded sets

of nucleotides are synthesized and assembled in vitro according to the methods described in Kay et al., 1993, Gene 128: 59-65.

The present invention further envisions utilization of libraries of vectors expressing a plurality of heterofunctional fusion proteins that are designed to form semirigid conformational structures.

The present invention further encompasses methods for preparing a protein, polypeptide and/or a peptide which binds to vinculin, dynein, or glutathione S-transferase comprising synthesizing, either chemically or by recombinant techniques, the amino acid sequence identified by screening a library of random peptides.

3.1. OBJECTS AND ADVANTAGES OF THE INVENTION

The present invention provides a method for identifying a binding molecule for vinculin, dynein, or glutathione S-transferase that is reproducible, quick, simple, efficient and relatively inexpensive. The invention provides a method of generating and screening a large library of diverse protein, polypeptide and/or peptide molecules to identify binding molecules for vinculin, dynein, or glutathione S-transferase. The diversity of binding characteristics that can be obtained with the methods and compositions of the present invention can be used in a wide variety of applications to mimic or replace naturally occurring binding molecules or portions thereof.

In contrast to methods that rely on isolation of specific genes and known sequences, the present invention has the advantage that there is no need for purifying or isolating genes nor any need for detailed knowledge of the function of portions of the binding sequence or the amino acids that are involved in ligand binding in order to produce a binding molecule having specificity for vinculin, dynein, or glutathione S-transferase. The only requirement is having the ligand needed to screen a peptide library to find a binding molecule with affinity for vinculin, dynein, or glutathione S-transferase. Since the peptide libraries are screened in vitro, the solvent

requirements involved in screening are not limited to aqueous solvents; thus, nonphysiological binding interactions and conditions different from those found in vivo can be exploited.

The binding molecules of the present invention, and, in some embodiments, TSARs or compositions comprising the binding domain of a TSAR (or a portion thereof having the same binding specificity) may be used in any in vivo or in vitro application that might make use of a peptide or polypeptide with the same binding specificity.

By virtue of their affinity for vinculin, dynein, or glutathione S-transferase, the binding molecules of the present invention, and, in some embodiments, TSARs or compositions comprising a TSAR binding domain having binding affinity for vinculin, dynein, or glutathione S-transferase or or a portion thereof can used in vivo to deliver a chemically or biologically active moiety to a specific target in or on the cell. The binding molecules or TSARs can also have in vitro a utility similar to monoclonal antibodies or other specific binding molecules for the detection, quantitation, separation or purification of vinculin, dynein, or glutathione S-transferase. In one embodiment, a number of binding molecules or TSARs or the binding domains thereof can be assembled as multimeric units to provide multiple binding domains that have the same specificity and can be fused to another molecule that has a biological or chemical activity.

The specific binding molecules identified according to the present invention are useful as inhibitors of enzymes. In particular, the enzyme may be glutathione S-transferase (GST).

The specific binding molecules of the present invention are also useful as reagents to detect and/or measure vinculin, dynein, and enzymes such as GST in cells or solutions.

4. BRIEF DESCRIPTION OF THE FIGURES

The present invention may be understood more fully by reference to the following detailed description of the invention, examples of specific embodiments of the invention and the appended figures in which:

Figure 1. Schematically illustrates construction of the TSAR-12 library. N = A, C, G or T; B = G, T or C and V = G, A or C. See text Section 6.2 for details. Insertion into a representative, appropriate vector and expression in an appropriate host is illustrated.
TTTTGTCGACN(NNB)$_{10}$NGCGGTG is SEQ ID NO 1.
TTTTACTAGT(VNN)$_{10}$VNCACCGC is SEQ ID NO 2.
TCGACN(NNB)$_{10}$NGCGGTG is SEQ ID NO 3.
CTAGT(VNN)$_{10}$VNCACCGC is SEQ ID NO 4.
SHSS(S/T)X$_{10}$$\phi$G$\delta$X$_{10}$SRPART is SEQ ID NO 5.

Figure 2. Schematically illustrates construction of the R8C library. See Section 6.3 for details.
TGACGTCTCGAGTTGT(NNK)$_8$TGTGGATCTAGAAGGATC is SEQ ID NO 6.
GATCCTTCTAGATCC is SEQ ID NO 7.
TCGAGTTGT(NNK)$_8$TGTGGAT is SEQ ID NO 8.
CTAGATCCACA(MNN)$_8$ACAAC is SEQ ID NO 9.
SSCX$_8$CGSRPSRT is SEQ ID NO 10.

Figure 3. A schematic depiction of the origin of one class of double-insert recombinants in the R8C library.
TCGAGTTGT(NNK)$_8$TGTGGATCTAGATCCACA(MNN)$_8$ACAAC is SEQ ID NO 11.
TCGAGTTGT(NNK)$_8$TGTGGATCTAGATCCACA(MNN)$_8$ACAAC is SEQ ID NO 12.

$SSCX_8CGSRSRTX_8TTR$ is SEQ ID NO 13.

Figure 4. Interaction of the vinculin binding phage Vin 12.1 with the fractions collected from an anion exchange column purification of chicken vinculin. The top panel shows the relative concentration of proteins in these fractions resolved by SDS-PAGE and stained with coomassie blue. Vinculin is the 130 kD band. The bottom panel shows the relative binding to each of the fractions, as detected by a phage ELISA kit (Pharmacia, Piscataway, NJ), of the vinculin binding phage Vin 12.1 and a random isolate from the R8C library (which does not bind vinculin) termed Nonvin 1.1. In the bottom panel: -■- = Vin 12.1; -o- = Nonvin 1.1.

Figure 5. Detection of vinculin by western blot using the vinculin binding peptide pVin 12.1 conjugated to streptavidin alkaline phosphatase (SaAP). The left panel shows the proteins present in the indicated vinculin purification column fractions and in a concentrated vinculin preparation (Vin). The right panel shows the interaction of SaAP conjugated peptide pVin 12.1 with these same proteins immobilized by western transfer. The peptide binds to vinculin and a 71 kD band but not to other bands present in the blot that were visualized by coomassie blue staining.

Figure 6. Comparison of binding of pVin 12.1 to binding of the SH3 domain binding peptide pA9. Binding was assayed to purified vinculin (vinc), whole cell lysates from CEF cells (cell), fraction 21 of Figure 2 (frc. 21), and a GST-SH3 fusion protein (SH3) immobilized by western transfer. pVin 12.1 binds to vinculin, the 71 kD protein, and some molecules in the whole cell lysate, but not to the GST-SH3 fusion protein. pA9 binds to the GST-SH3 fusion protein, the 71 kD protein, and some molecules in the whole cell lysate, but not to vinculin. This result indicates that pVin 12.1 binding to the 71 kD protein and to some molecules in the whole cell lysate is due to non-specific interactions.

Figure 7. Relative binding by ELISA of Vin 12.1 and Vin 12.1 G to R phage to fragments of vinculin. ■ Vin 12.1;  Vin 12 G to R. Below each pair of histograms, the fragment of vinculin that was used is indicated. *e.g.*, V431-850 indicates that the fragment consisting of the amino acids from position 431 to position 850 of vinculin was used.

Figure 8. pVin 12.1 competes with talin but not with paxillin or α-actinin for binding to immobilized vinculin. Vinculin was immobilized in the wells of an ELISA plate. Talin was preincubated with either no peptide, pVin 12.1, or pA9 and then added to the wells and incubated to allow binding of the talin to the immobilized vinculin. After the wells were washed, a mouse monoclonal antibody specific to talin was added, followed by goat anti-mouse IgG conjugated to alkaline phosphatase. Then alkaline phosphatase activity was determined by adding the reagent p-nitrophenyl phosphate and monitoring color development at 405 nm. The same procedure was carried out using paxillin and α-actinin except that mouse monoclonal antibodies specific to paxillin and α-actinin were used. In this assay, an inhibition of color production at 405 nm signifies competition for binding to vinculin between pVin 12.1 and talin, paxillin, or α-actinin. a = no competitor peptide; b = pVin 12.1 competitor peptide added; c = pA9 competitor peptide added. The data reported represent the average of the readings from three identical samples. The error bars show the range of the readings.

Figure 9. Effect of pVin 12.1 concentration on the talin-vinculin interaction. The experiment was performed as described in Figure 8 and shows peptide concentration dependent inhibition of binding. -■- pVin 12.1 competitor peptide added; -o- pA9 competitor peptide added.

Figure 10. The peptide p18 inhibits the activity of the enzyme GST. Shown are the results of experiments in which 100 μM (-●-), 50 μM (-▲-), 10 μM (-◆-), or 0 μM (-□-) of p18 was added to a reaction mixture of GST and its substrates, GSH and CDNB. (-⊠-) represents 100 μM of p15 added to a reaction mixture of GST and its substrates, GSH and CDNB.

Figure 11. GST-binding peptides can compete with GST-binding phage for binding to GST immobilized in the wells of microtiter plates. The inhibitory peptides used were: a = p23C; b = p23S; c = none.

Figure 12. p23 and p21 peptides can compete with biotinylated p23 and p21 for binding to GST immobilized in the wells of microtiter plates. The inhibitory peptides used were: a = 100 μM 23C; b = 100 μM 23S; c = 100 μM 21C; d = 100 μM 21S; e = no peptide.

Figure 13. Inhibition of the enzymatic activity of GST by GST-binding peptides. The inhibitory peptides used were: -■- no peptide; -●- 10 μM p23C; -▲- 10 μM p23S; -◆- 10 μM p15.

Figure 14. Inhibition of the enzymatic activity of GST by p21 peptides. The inhibitory peptides used were: -■- no peptide; -●- 10 μM p21C; -▲- 10 μM p21S; -◆- 10 μM p15.

Figure 15. Interaction of pVin 12.1 or the control peptide pC with whole vinculin isolated from chicken gizzard (Ck. Vin.), or with various fragments of vinculin expressed in E. coli. pC is a peptide that corresponds to the insert from a phage that specifically binds calmodulin and that was isolated from the R8C library (see Section 7.3.2). 0.01 μg of peptide (linked to biotin) and 1 μg of the appropriate vinculin or vinculin fragments

were incubated in 0.5 ml of wash buffer for one hour at room temperature; then 10 μl streptavidin-coated magnetic beads (PerSpective Diagnostics, Cambridge, MA) were added and incubated an additional hour. The beads were washed once with wash buffer and once with 1X PBS. The proteins bound to the beads were eluted in SDS-PAGE loading buffer and then resolved on an 8% polyacrylamide gel along with the input protein fragments. The proteins were visualized by coomassie blue. The results indicate that pVin 12.1 binds to the amino terminal 430 amino acids (compare the first panel from the left with the second) and to truncated fragments from the preparation of full-length vinculin in E. coli (see the fourth panel from the left). Since these proteins were isolated using a schistosomal GST-tag fused to the amino-terminus, all truncations most likely occur at the carboxy-terminus. Together, these results show that pVin 12.1 binds to a region of vinculin in the amino-terminal 430 amino acids only if the carboxy-terminus has been removed. Both pVin 12.1 and pC bind to BSA and the carboxy-terminus of vinculin, indicating that these interactions are non-specific.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for identifying and compositions identified which are proteins/polypeptides and/or peptides from random peptide libraries which bind to a ligand selected from among vinculin, dynein, or glutathione S-transferase. The peptide libraries may be chemically synthesized random peptide libraries or biological expression random peptide libraries. A random peptide library is a peptide library in which at any given position within the variable region of the peptides of the library it cannot be predicted which of the 20 naturally occurring amino acids will appear. In a particular embodiment, the library is a phage display random peptide library called a Totally Synthetic Affinity Reagent (TSAR) library in which the random peptide is between 20 and 200 amino acids in length. Such a TSAR library is described in Kay et al., 1993, Gene 128: 59-65.

As would be understood by those of skill in the art, the biological expression libraries for use according to the present invention comprise vectors containing variant nucleotide positions. The variant nucleotide positions have the potential to encode all 20 naturally occurring amino acids. The sequence of amino acids encoded by the variant nucleotides is unpredictable and substantially random in sequence. The terms "unpredicted", "unpredictable" and "substantially random" are used interchangeably in the present application with respect to the amino acids encoded and are intended to mean that at any given position within the binding domain encoded by the variant nucleotides it cannot be predicted which of the 20 naturally occurring amino acids will appear. In a specific embodiment, the libraries that are used encode peptides that have sequences that are not constructed to be homologous to a predetermined molecule known to bind the desired ligand.

The invention also includes determining the nucleotide sequence encoding the proteins/polypeptides and/or peptides from random peptide libraries which bind to a ligand selected from among vinculin, dynein, or glutathione S-transferase in order to deduce the amino acid sequence of the binding domain. When the amino acid sequences of a plurality of peptides that bind to a particular ligand have been deduced, it may be possible to identify a short sequence of amino acids which is common to or shared by a number of peptides binding that ligand, *i.e.*, shared binding motifs.

The methods of the present invention may employ any of the random peptide libraries that are known in the art. Such libraries are described in Section 2.1 and include chemically synthesized libraries as well as biological expression libraries, *e.g.*, libraries that employ a filamentous bacteriophage vector.

The peptide libraries used in the present invention may be libraries that are chemically synthesized *in vitro*. Examples of such libraries are given in Fodor et al., 1991, Science 251: 767-773, which describes the

synthesis of a known array of short peptides on an individual microscopic slide; Houghten et al., 1991, Nature 354: 84-86, which describes mixtures of free hexapeptides in which the first and second residues in each peptide were individually and specifically defined. Lam et al., 1991, Nature 354: 82-84, which describes a split synthesis scheme; Medynski, 1994, Bio/Technology 12: 709-710, describes split synthesis and T-bag synthesis methods as well. See also Gallop et al., 1994, J. Medicinal Chemistry 37: 1233-1251.

Screening to identify peptides from chemically synthesized random peptide libraries which bind to a ligand of choice can be carried out by methods well known in the art.

In a specific embodiment, the total number of unpredictable amino acids in the peptides of the chemical library used for screening is greater than or equal to 5 and less than or equal to 25; in other embodiments the total is in the range of 5-15 or 5-10 amino acids, preferably contiguous amino acids.

The methods of the present invention may also utilize biological expression libraries. Many suitable biological peptide libraries are known in the art and can be used. According to this approach, involving recombinant DNA techniques, peptides have been expressed in biological systems as either soluble fusion proteins or viral capsid fusion proteins.

A number of peptide libraries according to this approach have used the M13 phage. Although the N-terminus of the viral capsid protein, protein III (pIII), has been shown to be necessary for viral infection, the extreme N-terminus of the mature protein does tolerate alterations such as insertions. Accordingly, various peptide libraries, in which the diverse peptides are expressed as pIII fusion proteins, are known in the art; these libraries can be used in the present invention. Examples of such libraries are described below.

Scott and Smith, 1990, Science 249: 386-390 describe construction and expression of an "epitope library" of hexapeptides on the

surface of M13. The library was made by inserting a 33 base pair Bgl I digested oligonucleotide sequence into an Sfi I digested phage fd-tet, *i.e.*, fUSE5 RF. The 33 base pair fragment contains a random or "degenerate" coding sequence $(NNK)_6$ where N represents G, A, T or C and K represents G or T.

Cwirla et al., 1990, Proc. Natl. Acad. Sci. USA 87: 6378-6382 also described a somewhat similar library of hexapeptides expressed as pIII gene fusions of M13 fd phage. PCT publication WO 91/19818 dated December 26, 1991 by Dower and Cwirla describes a similar library of pentameric to octameric random amino acid sequences.

Devlin et al., 1990, Science, 249: 404-406, describes a peptide library of about 15 residues generated using an (NNS) coding scheme for oligonucleotide synthesis in which S is G or C.

Christian and colleagues have described a phage display library, expressing decapeptides (Christian et al., 1992, J. Mol. Biol. 227: 711-718).

The biological peptide libraries discussed above are meant to be illustrative and not limiting. It will be recognized by one of skill in the art that many other biological peptide libraries disclosed in various publications may be suitable for use in the practice of the present invention.

In a particular embodiment, the methods of the present invention employ phage display libraries encoding Totally Synthetic Affinity Reagents (TSARs). Such libraries are known as TSAR libraries. An example of a TSAR library is found in Kay et al., 1993, Gene 128: 59-65. As used in the present invention, a TSAR is intended to encompass a concatenated heterofunctional protein, polypeptide and/or peptide that includes at least two distinct functional regions. One region of the heterofunctional TSAR molecule is a binding domain with affinity for a ligand, that is preferably characterized by 1) its strength of binding under specific conditions, 2) the stability of its binding under specific conditions, and 3) its selective specificity for the chosen ligand. A second region of the heterofunctional TSAR

molecule is an effector domain that is biologically or chemically active to enhance expression and/or detection and/or purification of the TSAR. The effector domain is chosen from a number of biologically or chemically active proteins including a structural protein or fragment that is accessibly expressed as a surface protein of a vector, an enzyme or fragment thereof, a toxin or fragment thereof, a therapeutic protein or peptide, or a protein or peptide whose function is to provide a site for attachment of a substance such as a metal ion, etc., that is useful for enhancing expression and/or detection and/or purification of the expressed TSAR.

According to one embodiment of the invention, a TSAR can contain an optional additional linker domain or region between the binding domain and the effector domain. The linker region serves (1) as a structural spacer region between the binding and effector domains; (2) as an aid to uncouple or separate the binding and effector domains; or (3) as a structural aid for display of the binding domain and/or the TSAR by the expression vector.

In another embodiment, the present invention utilizes "constrained", "structured" or "semi-rigid" random peptide libraries. Typically, these libraries express peptides that are substantially random but contain a small percentage of fixed residues within or flanking the random sequences that have the result of conferring structure or some degree of conformational rigidity to the peptide. In a semirigid peptide library, the plurality of synthetic oligonucleotides express peptides that are each able to adopt only one or a small number of different conformations that are constrained by the positioning of codons encoding certain structure conferring amino acids in or flanking the synthesized variant or unpredicted oligonucleotides. Unlike linear, unconstrained libraries in which the plurality of proteins expressed potentially adopt thousands of short-lived different conformations, in a semirigid peptide library, the plurality of proteins expressed can adopt only a single or a small number of conformations. Such

semi-rigid libraries, if biological expression libraries, may contain a binding domain, an effector domain, and an optional linker domain, as described above for TSAR libraries.

In this invention, the ligand is vinculin, dynein, or an enzyme such as glutathione S-transferase.

The present invention provides binding molecules for vinculin, dynein, or glutathione S-transferase and compositions comprising such binding molecules. The present invention further provides novel TSAR reagents as well as compositions comprising a binding domain of a TSAR or a portion thereof which has specificity for a ligand selected from among vinculin, dynein, or glutathione S-transferase and methods for using TSARs and compositions comprising a binding domain of a TSAR or a portion thereof which retains the binding specificity of the TSAR binding domain.

Also provided by the present invention is a class of peptides which bind specifically to vinculin. In a particular embodiment, the class of peptides contains peptides which have the amino acid sequences encompassed by the formula:

$X_1X_2X_3X_4X_5VX_6X_7X_8ARX_9VX_{10}X_{11}ALTX_{12}TLX_{13}A$ (SEQ ID NO 14)

where X_1 is G, R or S;

X_2 is G or E;

X_3 is F or L;

X_4 is D, V, A, H, or E;

X_5 is D, L, Y, or N;

X_6 is Y or F;

X_7 is D, Y, H, or A;

X_8 is W or L;

X_9 is G, R, E, or A;

X_{10} is S or T;

X_{11} is S or A;

X_{12} is T or A; and

X_{13} is V or L

where the single letters represent the well-known one-letter symbols for the amino acids.

More preferably, the amino acid sequence is:

$X_1X_2X_3X_4X_5VX_6X_7X_8ARX_9VX_{10}X_{11}ALTX_{12}TLX_{13}A$ (SEQ ID NO 14)

where X_1 is G;

X_2 is G or E;

X_3 is F;

X_4 is D, V, or A;

X_5 is D or L;

X_6 is Y or F;

X_7 is D, Y, or H;

X_8 is W;

X_9 is G, R, E, or A;

X_{10} is S or T;

X_{11} is S;

X_{12} is T or A; and

X_{13} is V or L.

Also provided by the present invention is a class of peptides having 5 to 50 amino acid residues including an amino acid sequence of a formula selected from the group consisting of

X_1VX_2,

ARX_3VX_4 (SEQ ID NO 85), and

LTX_5TL (SEQ ID NO 86),

said amino acid sequence being positioned anywhere along the peptide, in which X_1 is D, L, Y, or N; X_2 is Y or F; X_3 is R, G, E, or A; X_4 is S or T; X_5 is T or A; in which said peptide specifically binds vinculin. Such peptides may have one, two, or all three of the sequences of the above formulas.

The present invention also provides peptides which bind specifically to glutathione S-transferase. In a particular embodiment, such peptides have the amino acid sequence:

CX_aWDGX_bC (SEQ ID NO 15)

where X is W, S, E, G, Q, A, P, F, M, I, K, L, Y, N, T, or D; and $a + b = 5$. In such peptides, the amino acid sequence WDG is flanked on either side by a total of 5 additional amino acids, *e.g.*, XXWDGXXX, XWDGXXXX, WDGXXXXX, etc. Such peptides may be linear peptides or may be cyclized via disulfide bond formation between the cysteines.

In addition, the present invention also provides a peptide which binds specifically to glutathione S-transferase, in which the peptide has the amino acid sequence:

$CX_1X_2X_3X_4LGX_5X_6C$ (SEQ ID NO 16)

where X_1 is M or L;

X_2 is G or D;

X_3 is D or E;

X_4 is N, S, or D;

X_5 is W, K, Q, or D; and

X_6 is D, S, G, or M.

Such peptides may be linear peptides or may be cyclized via disulfide bond formation between the cysteines.

The present invention also provides a peptide that specifically binds dynein having the amino acid sequence WVMLGYCAKAGGAHRDRMRTAIC (SEQ ID NO 20).

5.1. METHODS TO IDENTIFY BINDERS: CONSTRUCTION OF LIBRARIES

In its most general embodiment, the process of the present method for rapidly and efficiently identifying protein/polypeptide and/or peptides that specifically bind to vinculin, dynein, or glutathione S-transferase involves screening a random peptide library by contacting the library with a ligand selected from among vinculin, dynein, or glutathione S-transferase to identify members of the library that specifically bind to the ligand.

In a particular embodiment, the process of the present method utilizes TSAR libraries and comprises screening a library of recombinant vectors expressing inserted synthetic oligonucleotide sequences encoding a plurality of proteins, polypeptides and/or peptides as fusion proteins, for example, attached to an accessible surface structural protein of a vector to isolate those members producing proteins, polypeptides and/or peptides that bind to vinculin, dynein, or glutathione S-transferase. The nucleic acid sequence of the inserted synthetic oligonucleotides of the isolated vector is determined and the amino acid sequence encoded is deduced to identify a TSAR binding domain that binds the ligand of choice (vinculin, dynein, or glutathione S-transferase). TSAR libraries that can be used in the screening steps of the present invention include, but are not limited to, the TSAR-9 library disclosed in Kay et al., 1993, Gene 128: 59-65 as well as the TSAR-12 library described in Section 6.2 and Figure 1. Alternatively, if such a library expressing totally synthetic peptides, preferably random peptides, is not readily available, a TSAR library of recombinant vectors can be constructed by methods well known in the art (see also discussion hereinbelow).

The present invention encompasses a method for identifying a protein, polypeptide and/or peptide which binds to a ligand selected from among vinculin, dynein, or glutathione S-transferase comprising: screening a library of random peptides, preferably by screening a library of recombinant vectors which express a plurality of heterofunctional fusion proteins

comprising (a) a binding domain encoded by an oligonucleotide comprising unpredictable nucleotides in which the unpredictable nucleotides are arranged in one or more contiguous sequences, wherein the total number of unpredictable nucleotides is greater than or equal to 60 and less than or equal to 600, and (b) an effector domain encoded by an oligonucleotide sequence encoding a protein or peptide that enhances expression or detection of the binding domain, by contacting the plurality of heterofunctional fusion proteins with the ligand under conditions conducive to ligand binding and isolating the fusion proteins which bind to the ligand. Alternatively, the present invention encompasses a method for identifying a protein and/or peptide which binds to a ligand selected from among vinculin, dynein, or glutathione S-transferase comprising: (a) generating a library of vectors expressing a plurality of heterofunctional fusion proteins comprising (i) a binding domain encoded by a double stranded oligonucleotide comprising unpredictable nucleotides in which the unpredictable nucleotides are arranged in one or more contiguous sequences, wherein the total number of unpredictable nucleotides is greater than or equal to 60 and less than or equal to 600, and (ii) an effector domain encoded by an oligonucleotide sequence encoding a protein or peptide that enhances expression or detection of the binding domain; and (b) screening the library of vectors by contacting the plurality of heterofunctional fusion proteins with the ligand under conditions conducive to ligand binding and isolating the heterofunctional fusion protein which binds to the ligand. Additionally, the methods or the invention further comprise determining the nucleotide sequence encoding the binding domain of the heterofunctional fusion protein identified to deduce the amino acid sequence of the binding domain.

In another embodiment, the present invention encompasses a method for identifying a protein, polypeptide and/or peptide which binds to a ligand selected from among vinculin, dynein, or glutathione S-transferase comprising screening a rigid or semi-rigid library (*e.g.*, the R8C library described in Section 6.3 and Figures 2 and 3) by contacting the library with the ligand under conditions conducive to ligand binding and isolating the members of the library which bind to the ligand.

5.1.1. SYNTHESIS AND ASSEMBLY OF OLIGONUCLEOTIDES

In a particular embodiment, in order to prepare a library of vectors expressing a plurality of protein, polypeptide and/or peptide TSARs, single stranded sets of oligonucleotides may be synthesized and assembled in vitro according to the procedures that are described in Kay et al., 1993, Gene 128: 59-65. Alternatively, to prepare a TSAR library, single stranded sets of oligonucleotides may be synthesized and assembled in vitro according to the procedures that are described in Section 6.2 and Figure 1.

To prepare a semi-rigid library, single stranded sets of oligonucleotides may be synthesized and assembled in vitro according to the procedures that are described in Section 6.3 and Figures 2 and 3.

5.1.2. INSERTION OF SYNTHETIC OLIGONUCLEOTIDES INTO AN APPROPRIATE VECTOR

The plurality of oligonucleotides of appropriate size prepared as described in Kay et al., 1993, Gene 128: 59-65 or according to the procedures that are described in Section 6.2 or 6.3 and Figures 1, 2, and 3 is inserted into an appropriate vector which, when inserted into a suitable host, expresses the plurality of proteins, polypeptides and/or proteins as heterofunctional fusion proteins with an expressed component of the vector which are screened to identify TSARs, or, in the case of the R8C library, binding molecules, having affinity for a ligand selected from among vinculin, dynein, or glutathione S-transferase. The plurality of proteins, polypeptides and/or

peptides may further comprise a linker domain between the binding and effector domains. The linker domain may be expressed as a fusion protein with the effector domain of the vector into which the plurality of oligonucleotides are inserted.

5.1.2.1. LINEAR LIBRARIES

The skilled artisan will recognize that, when utilizing biological expression libraries, to achieve transcription and translation of the plurality of oligonucleotides, the synthetic oligonucleotides must be placed under the control of a promoter compatible with the chosen vector-host system. A promoter is a region of DNA at which RNA polymerase attaches and initiates transcription. The promoter selected may be any one that has been synthesized or isolated that is functional in the vector-host system. For example, E. coli, a commonly used host system, has numerous promoters such as the lac or trp promoter or the promoters of its bacteriophages or its plasmids. Also synthetic or recombinantly produced promoters such as the p_{TAC} promoter may be used to direct high level expression of the gene segments adjacent to it.

Signals are also necessary in order to attain efficient translation of the inserted oligonucleotides. For example in E. coli mRNA, a ribosome binding site includes the translational start codon AUG or GUG in addition to other sequences complementary to the bases of the 3′ end of 16S ribosomal RNA. Several of these latter sequences such as the Shine/Dalgarno (S/D) sequence have been identified in E. coli and other suitable host cell types. Any S/D-ATG sequence which is compatible with the host cell system can be employed.

Any of a variety of vectors can be used including, but not limited to, bacteriophage vectors such as ØX174, λ, M13 and its derivatives, f1, fd, Pf1, etc., phagemid vectors, plasmid vectors, insect viruses, such as baculovirus vectors, mammalian cell vectors, including such as parvovirus

vectors, adenovirus vectors, vaccinia virus vectors, retrovirus vectors, etc., yeast vectors such as Ty1, killer particles, etc.

In the case of TSAR libraries, an appropriate vector contains or is engineered to contain a gene encoding an effector domain of a TSAR to aid expression and/or detection of the TSAR. The effector domain gene contains or is engineered to contain multiple cloning sites. At least two different restriction enzyme sites within such gene, comprising a polylinker, are preferred. The vector DNA is cleaved within the polylinker using two different restriction enzymes to generate termini complementary to the termini of the double stranded synthesized oligonucleotides assembled as described above. Preferably the vector termini after cleavage have or are modified, using DNA polymerase, to have non-compatible sticky ends that do not self-ligate, thus favoring insertion of the double-stranded synthesized oligonucleotides and hence formation of recombinants expressing the TSAR fusion proteins, polypeptides and/or peptides. The double stranded synthesized oligonucleotides are ligated to the appropriately cleaved vector using DNA ligase.

The vector may be or may be derived from a filamentous bacteriophage, including but not limited to M13, f1, fd, Pf1, etc. vector encoding a phage structural protein, preferably a phage coat protein, such as pIII, pVIII, etc. The filamentous phage may be an M13-derived phage vector such as m655, m663 and m666 described in Fowlkes et al., 1992, BioTechniques, 13:422-427 which encodes the structural coat protein pIII.

The phage vector is chosen to contain or is constructed to contain a cloning site located in the 5′ region of a gene encoding a bacteriophage structural protein so that the plurality of synthesized double stranded oligonucleotides inserted are expressed as fusion proteins on the surface of the bacteriophage. This advantageously provides not only a plurality of accessible expressed proteins/peptides but also provides a physical link between the proteins/peptides and the inserted oligonucleotides to provide

for easy screening and sequencing of the identified TSARs. Alternatively, the vector is chosen to contain or is constructed to contain a cloning site near the 3′ region of a gene encoding a structural protein so that the plurality of expressed proteins constitute C-terminal fusion proteins.

The structural protein may be the bacteriophage protein pIII. The m663 vector described by Fowlkes containing the pIII gene having a c-myc-epitope comprising a "stuffer fragment" introduced at the N-terminal end, flanked by Xho I and Xba I restriction sites is an example of a suitable vector that utilizes the pIII gene. The library is constructed by cloning the plurality of synthesized oligonucleotides into a cloning site near the N-terminus of the mature coat protein of the appropriate vector, preferably the pIII protein, so that the oligonucleotides are expressed as coat protein-fusion proteins.

Alternatively, the plurality of oligonucleotides may be inserted into a phagemid vector. Phagemids are utilized in combination with a defective helper phage to supply missing viral proteins and replicative functions. Helper phage useful for propagation of M13 derived phagemids as viral particles include but are not limited to M13 phage K07, R408, VCS, etc. Generally, according to a preferred mode of this embodiment, the appropriate phagemid vector was constructed by engineering the Bluescript II SK+ vector (GenBank #52328) (Alting-Mees et al., 1989, Nucl. Acid Res. 17(22):p 9494); to contain (1) a truncated portion of the M13 pIII gene, *i.e.*, nucleotides encoding amino acid residues 198-406 of the mature pIII, (2) the PelB signal sequence including an upstream ribosome binding site and a short polylinker of Pst I, Xho I, Hind III, and Xba I restriction sites, in which the Xho I and Xba I sites are positioned so the synthesized double stranded oligonucleotides could be cloned and expressed in the same reading frame as the m663 phage vector; and (3) the linker sequence encoding gly-gly-gly-gly-ser between the polylinker and the pIII gene.

The double-stranded DNA segment encoding the PelB signal sequence was prepared by PCR from *E. coli* DNA using the oligonucleotides

GCGACGCGACGAGCTCGACTGCAAATTCTATTTCAA (SEQ ID NO 17) and

CTAATGTCTAGAAAGCTTCTCGAGCCCTGCAGCTGCACCTGGGCCAT CGACTGG (SEQ ID NO 18). The termini of the PCR product introduced a short polylinker of Pst I, Xho I, Hind III, and Xba I sites into the vector.

Alternatively, the synthesized oligonucleotides are inserted into a plasmid vector.

5.1.2.2. STRUCTURED LIBRARIES

The present invention may utilize libraries of vectors expressing a plurality of heterofunctional fusion proteins that are designed to form semirigid conformational structures. This is accomplished by incorporating into the synthesized nucleotide sequences additional invariant residues flanking contiguous sequences of variant nucleotides. These additional invariant nucleotide sequences are designed to encode amino acids that will confer structure in the binding domain of the expressed heterofunctional fusion protein. In a preferred embodiment, the additional invariant nucleotides code for cysteine residues in which the cysteine residues are separated from each other by 8 or 9 amino acid residues. When the library is expressed in an oxidizing environment, at least one disulfide bond is formed, thereby allowing for the formation of at least one loop or even a cloverleaf conformation in each heterofunctional fusion protein.

5.1.3. EXPRESSION OF VECTORS

Once the appropriate expression vectors are prepared, they are inserted into an appropriate host, such as E. coli, Bacillus subtilis, insect cells, mammalian cells, yeast cells, etc., for example by electroporation, and the plurality of oligonucleotides is expressed by culturing the transfected host cells under appropriate culture conditions for colony or phage production.

Preferably, the host cells are protease deficient, and may or may not carry suppressor tRNA genes.

A small aliquot of the electroporated cells are plated and the number of colonies or plaques are counted to determine the number of recombinants. The library of recombinant vectors in host cells is plated at high density for a single amplification of the recombinant vectors.

For example, the use of recombinant M13 vector m666, m655 or m663, engineered to contain the synthesized double stranded oligonucleotides, is described in Fowlkes et al., 1992, BioTechniques, 13:422-427.

TSARs can be expressed in a plasmid vector contained in bacterial host cells such as E. coli. The TSAR proteins accumulate inside the E. coli cells and a cell lysate is prepared for screening.

Phagemid vectors containing the synthesized double stranded oligonucleotides, expressed on the outer surface of the extruded phage, are propagated either as plasmids in bacteria or as bacteriophage with helper phage.

5.2. METHODS TO IDENTIFY BINDERS: SCREENING LIBRARIES

Once a suitable random peptide library has been constructed (or otherwise obtained), the library is screened to identify peptides having binding affinity for vinculin, dynein, or glutathione S-transferase. Screening the libraries can be accomplished by any of a variety of methods known to those of skill in the art. See, e.g., the following references, which disclose screening of peptide libraries: Parmley and Smith, 1989, Adv. Exp. Med. Biol. 251: 215-218; Scott and Smith, 1990, Science 249: 386-390; Fowlkes et al., 1992; BioTechniques 13: 422-427; Oldenburg et al., 1992, Proc. Natl. Acad. Sci. USA 89: 5393-5397; Yu et al., 1994, Cell 76: 933-945; Staudt et al., 1988, Science 241: 577-580; Bock et al., 1992, Nature 355: 564-566; Tuerk et al., 1992, Proc. Natl. Acad. Sci. USA 89: 6988-6992; Ellington et

al., 1992, Nature 355: 850-852; U.S. Patent No. 5,096,815, U.S. Patent No. 5,223,409, and U.S. Patent No. 5,198,346, all to Ladner et al.; and Rebar and Pabo, 1993, Science 263: 671-673. See also PCT publication WO 94/18318, dated August 18, 1994.

One of ordinary skill in the art will recognize that, with suitable modifications, the screening methods described below would be suitable for a wide variety of biological expression libraries, *e.g.*, the TSAR and the R8C libraries described herein.

Once a library has been constructed or otherwise obtained, the library is screened to identify binding molecules having binding affinity for a ligand selected from among vinculin, dynein, or an enzyme such as, but not limited to, glutathione S-transferase. In this invention, a ligand is a substance that specifically interacts with the binding domain of a peptide from a library and is selected from the group consisting of vinculin, dynein, or enzymes, *e.g.*, glutathione S-transferase.

In an embodiment of the invention directed to a method of identifying an inhibitor of an enzyme, the enzyme may be *e.g.*, glutathione S-transferase, superoxide dismutase, a protein kinase, DNA polymerase, RNA polymerase, thioredoxin, glyceraldehyde 3-phosphate dehydrogenase, a protein phosphatase, a viral protease, acetylcholinesterase, an enzyme involved in DNA repair, a DNA topoisomerase, plasmin, dihydrofolate reductase, trypsin, chymotrypsin, pepsin, or others.

Screening the libraries can be accomplished by any of a variety of methods known to those of skill in the art. Screening methods are described in Fowlkes et al., 1992, BioTechniques, 13:422-427 and include contacting the vectors with an immobilized target ligand and harvesting those vectors that bind to said ligand. Such useful screening methods, are designated "panning" methods. In panning methods useful to screen the present libraries, the target ligand can be immobilized on plates, beads (such as magnetic beads), sepharose, beads used in columns, etc. If desired, the

immobilized target ligand can be "tagged", *e.g.*, using such as biotin, fluoroscein isothiocyanate, rhodamine, etc. *e.g.* for FACS sorting. Panning is also disclosed in Parmley, S.F. and Smith, G.P., 1988, Gene 73: 305-318.

Since the peptide libraries are screened in vitro, the solvent requirements involved in screening are not limited to aqueous solvents; thus, nonphysiological binding interactions and conditions different from those found in vivo can be exploited.

Screening a library can be achieved using a method comprising a first "enrichment" step and a second filter lift as follows.

Binders from an expressed library (*e.g.*, in phage) capable of binding to a given ligand ("positives") are initially enriched by one or two cycles of panning or affinity chromatography. A microtiter well is passively coated with the ligand (*e.g.*, about 10 μg in 100 μl). The well is then blocked with a solution of BSA to prevent non-specific adherence of the phage of the library to the plastic surface. For example, about 10^{11} phage particles expressing peptides are then added to the well and incubated for several hours. Unbound phage are removed by repeated washing of the plate, and specifically bound phage are eluted using an acidic glycine-HCl solution or other elution buffer. The eluted phage solution is neutralized with alkali, and amplified, *e.g.*, by infection of E. coli and plating on large petri dishes containing broth in agar. Amplified cultures expressing the binding peptides are then titered and the process repeated. Alternatively, the ligand can be covalently coupled to agarose or acrylamide beads using commercially available activated bead reagents. The phage solution is then simply passed over a small column containing the coupled bead matrix which is then washed extensively and eluted with acid or other eluant. In either case, the goal is to enrich the positives to a frequency of about $> 1/10^5$.

Following enrichment, a filter lift assay is conducted. For example, when specific binders are expressed in phage, approximately 1-2 x 10^5 phage are added to 500 μl of log phase E. coli and plated on a large Luria

Broth-agarose plate with 0.7% agarose in broth. The agarose is allowed to solidify, and a nitrocellulose filter (*e.g.*, 0.45 μ) is placed on the agarose surface. A series of registration marks is made with a sterile needle to allow re-alignment of the filter and plate following development as described below. Phage plaques are allowed to develop by overnight incubation at 37 °C (the presence of the filter does not inhibit this process). The filter is then removed from the plate with phage from each individual plaque adhered in situ. The filter is then exposed to a solution of BSA or other blocking agent for 1-2 hours to prevent non-specific binding of the ligand (or "probe").

The probe itself is labeled, for example, either by biotinylation (using commercial NHS-biotin) or direct enzyme labeling, *e.g.*, with horse radish peroxidase or alkaline phosphatase. Probes labeled in this manner are indefinitely stable and can be re-used several times. The blocked filter is exposed to a solution of probe for several hours to allow the probe to bind in situ to any phage on the filter displaying a peptide with significant affinity to the probe. The filter is then washed to remove unbound probe, and then developed by exposure to enzyme substrate solution (in the case of directly labeled probe) or further exposed to a solution of enzyme-labeled avidin (in the case of biotinylated probe). Positive phage plaques are identified by localized deposition of colored enzymatic cleavage product on the filter which corresponds to plaques on the original plate. The developed filter is simply realigned with the plate using the registration marks, and the "positive" plaques are cored from the agarose to recover the phage. Because of the high density of plaques on the original plate, it is usually impossible to isolate a single plaque from the plate on the first pass. Accordingly, phage recovered from the initial core are re-plated at low density and the process is repeated to allow isolation of individual plaques and hence single clones of phage.

Successful screening experiments are optimally conducted using 3 rounds of serial screening. The recovered cells are then plated at a low density to yield isolated colonies for individual analysis. The individual

colonies are selected and used to inoculate LB culture medium containing ampicillin. After overnight culture at 37°C, the cultures are then spun down by centrifugation. Individual cell aliquots are then retested for binding to the target ligand attached to the beads. Binding to other beads, having attached thereto a non-relevant ligand, can be used as a negative control.

One important aspect of screening the libraries is that of elution. The following discussion is applicable to any system where the random peptide is expressed on a surface fusion molecule. It is conceivable that the conditions that disrupt the peptide-target interactions during recovery of the phage are specific for every given peptide sequence from a plurality of proteins expressed on phage. For example, certain interactions may be disrupted by acid pH's but not by basic pH's, and *vice versa*. Thus, it may be desirable to test a variety of elution conditions (including but not limited to pH 2-3, pH 12-13, excess target in competition, detergents, mild protein denaturants, urea, varying temperature, light, presence or absence of metal ions, chelators, etc.) and compare the primary structures of the binding proteins expressed on the phage recovered for each set of conditions to determine the appropriate elution conditions for each ligand/binding protein combination. Some of these elution conditions may be incompatible with phage infection because they are bactericidal and will need to be removed by dialysis (*i.e.*, dialysis bag, Centricon/Amicon microconcentrators).

According to one method of screening, the libraries expressing binding proteins as a surface protein of either a vector or a host cell, *e.g.*, phage or bacterial cell, can be screened by passing a solution of the library over a column of a ligand immobilized to a solid matrix, such as sepharose, silica, etc., and recovering those phage that bind to the column after extensive washing and elution.

According to yet another embodiment, weak binding library members can be isolated based on a retarded chromatographic properties. According to one mode of this embodiment for screening, fractions are

collected as they come off the column, saving the trailing fractions (*i.e.*, those members that are retarded in mobility, relative to the peak fraction are saved). These members are then concentrated and passed over the column a second time, again saving the retarded fractions. Through successive rounds of chromatography, it is possible to isolate those that have some affinity, albeit weak, to the immobilized ligand. These library members are retarded in their mobility because of the millions of possible ligand interactions as the member passes down the column. In addition, this methodology selects those members that have modest affinity to the target, and which also have a rapid dissociation time. If desired, the oligonucleotides encoding the binding domain selected in this manner can be mutagenized, expressed and rechromatographed (or screened by another method) to discover improved binding activity.

In particular, saturation mutagenesis can be carried out using synthetic oligonucleotides synthesized from "doped" nucleotide reservoirs. The doping is carried out such that the original peptide sequence is represented only once in 10^6 unique clones of the mutagenized oligonucleotide. The assembled oligonucleotides are cloned into a parental vector. Preferably, the vector is m663 (Fowlkes et al., 1992, BioTechniques 13: 422-427).

5.3. BINDERS AND COMPOSITIONS COMPRISING A BINDING DOMAIN

Although framed in terms of TSARs, the following discussion will be understood by one of ordinary skill in the art to be applicable to other libraries as well, *e.g.*, the R8C library described hereinbelow.

In one embodiment of the present invention, novel totally synthetic affinity reagents called TSARs are identified which can be produced as soluble, easily purified proteins/polypeptides and/or peptides that can be made and isolated in commercial quantities. These TSAR reagents are concatenated heterofunctional proteins, polypeptides and/or peptides that

include at least two distinct functional regions. One region of the heterofunctional TSAR molecule is a binding domain with affinity for a ligand that is preferably characterized by 1) its strength of binding under specific conditions, 2) the stability of its binding under specific conditions, and 3) its selective specificity for the chosen ligand. A second region of the heterofunctional TSAR molecule is an effector domain that is biologically or chemically active to enhance expression and/or detection of the TSAR. The effector domain is chosen from a number of biologically or chemically active proteins including a structural protein that is accessibly expressed as a surface protein of a vector, an enzyme or fragment thereof, a toxin or fragment thereof, a therapeutic protein or peptide or a protein or a peptide whose function is to provide a site for attachment of a substance such as a metal ion, etc., that is useful for enhancing expression and/or detection of the expressed TSAR. In a preferrred embodiment, the binding domain has affinity for vinculin, dynein, or glutathione S-transferase.

A TSAR can contain an optional additional region, *i.e.*, a linker domain between the binding domain and the effector domain. The linker region serves (1) as a structural spacer region between the binding and effector domains; (2) as an aid to uncouple or separate the binding and effector domains; or (3) as a structural aid for display of the binding domain and/or the TSAR by the expression vector. The linker sequence can be stable and provide for separation of the TSAR regions or it can be selectively susceptible to cleavage by chemical, biological, physical or enzymatic means. If a cleavable linker is used, the sequence employed is one that allows the binding domain portion of the TSAR to be released from the effector domain of the TSAR protein. Thus when a linker is used that is susceptible to cleavage, the heterofunctional TSAR protein can be an intermediate in the production of a unifunctional binding protein, polypeptide or peptide having the same binding specificity as the TSAR.

The cleavable sequence may be one that is enzymatically degradable. Among the useful sequences that can be used as an enzymatically cleavable linker domain are those which are susceptible to cleavage by collagenase, enterokinase, Factor Xa, thrombin, microbial proteases, peptidases, viral proteases, the complement cascade enzymes or enzymes of the blood coagulation/clot dissolution pathway

Alternatively, the linker portion can be stable or impervious to chemical and/or enzymatic cleavage and serve as a link between the binding domain and the other peptide portion(s) of the TSAR. For example, the linker domain can be a deformable protein moiety which can serve as a shape-controllable aid for recovery of the binding domain during elution. As another example, the linker domain can provide a (a) hinge or link region, such as provided by one or more proline residues; (b) a swivel region, such as provided by one or more glycine residues; or (c) a heterodimerization domain such as provided by a c-fos or c-jun sequence which aid in displaying the TSAR binding domains in the form of bimolecular pockets.

The chemically or biologically active effector domain of the TSAR may impart detectable, diagnostic, enzymatic or therapeutic characteristics to the TSAR. The enzymatic activity or therapeutic activity may be useful in identifying or detecting the TSAR during the screening process as well as being useful, *e.g.*, for therapeutic effects where the TSAR is employed in an in vivo application. Alternatively, the effector domain can be a protein moiety that binds a metal, including but not limited to radioactive, magnetic, paramagnetic, etc. metals, and allows detection of the TSAR.

There is no intended specified order for the two or more regions of the TSAR relative to each other except that the linker domain, if present, must be between the binding domain and the effector domain of the TSAR. The positions of the regions of the TSAR are otherwise interchangeable. The binding domain may be located at the N-terminal end of

the heterofunctional protein, polypeptide or peptide and the effector domain may be located at the carboxyl terminal end.

The TSAR can include multiple binding domains or multiple active effector portions or combinations of multiples of each.

Once a TSAR binding a ligand selected from among vinculin, dynein, or glutathione S-transferase has been identified by the method of the invention, the amino acid sequence of the binding domain of the TSAR can be deduced from the nucleotide sequence of the inserted oligonucleotide sequence in the vector identified as expressing the TSAR. The protein/peptide comprising the binding domain of the TSAR can be produced either by recombinant DNA techniques or synthesized by standard chemical methods known in the art (*e.g.*, see Hunkapiller et al., 1984, Nature 310: 105-111). Whether produced by recombinant or chemical synthetic techniques, the proteins/peptides comprising the binding domain of the identified TSAR include those having an amino acid sequence identical to the TSAR binding domain as well as those in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the non-polar (hydrophobic) amino acids include glycine, alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic and glutamic acid.

When a TSAR has been identified as a binder for a particular target ligand of interest, it may be useful to determine what region(s) of the

expressed TSAR peptide sequence is (are) responsible for binding to the target ligand. Such analysis can be conducted at two different levels, *i.e.*, the nucleotide sequence and amino acid sequence levels.

By molecular biological techniques it is possible to verify and further analyze a ligand binding TSAR at the level of its oligonucleotides. First, the oligonucleotides of the insert can be cleaved using appropriate restriction enzymes and religated into the original expression vector and the expression product of such vector screened for ligand binding to verify that the TSAR oligonucleotides encode the binding peptide. Second, the oligonucleotides can be transferred into another vector. The newly expressed fusion proteins should acquire the same binding activity if the domain is necessary and sufficient for binding to the ligand. This last approach also assesses whether or not flanking amino acid residues encoded by the original vector (*i.e.*, fusion partner) influence TSAR peptide in any fashion. Third, the oligonucleotides can be synthesized, based on the nucleotide sequence determined for the TSAR, amplified by cloning or PCR amplification using internal and flanking primers cleaved into two pieces and cloned as two half-TSAR fragments. In this manner, the inserted oligonucleotides are subdivided into two equal halves. If the TSAR domain important for binding is small, then one recombinant clone would demonstrate binding and the other would not. If neither have binding, then either both are important or the essential portion of the domain spans the middle (which can be tested by expressing just the central region).

Alternatively, by synthesizing peptides corresponding to the predicted TSAR peptide, the binding domains can be analyzed. First, the entire peptide should be synthesized and assessed for binding to the target ligand to verify that the TSAR peptide is necessary and sufficient for binding. Second, short peptide fragments, for example, overlapping 10-mers, can by synthesized, based on the amino acid sequence of the TSAR binding domain, and tested to identify those binding the ligand.

In addition, in certain instances, linear motifs may become apparent after comparing the primary structures of different TSARs having binding affinity for a target ligand. The contribution of these motifs to binding can be verified with synthesized peptides in competition experiments (*i.e.*, determine the concentration of peptide capable of inhibiting 50% of the binding of the phage to its target; IC_{50}). Conversely, the motif or any region suspected to be important for binding can be removed or mutated from the DNA encoding the TSAR insert and the altered displaced peptide can be retested for binding.

Furthermore, once the binding domain of a TSAR has been identified, new TSARs can be created by isolating and fusing the binding domain of one TSAR to a different effector domain. The biologically or chemically active effector domain of the TSAR can thus be varied. Alternatively, the binding characteristics of an individual TSAR can be modified by varying the TSAR binding domain sequence to produce a related family of TSARs with differing properties for a specific ligand.

Moreover, in a method of directed evolution, the identified TSAR proteins/peptides can be improved by additional rounds of mutagenesis, selection, and amplification of the nucleotide sequences encoding the TSAR binding domains. Mutagenesis can be accomplished by creating and cloning a new set of oligonucleotides that differ slightly from the parent sequence, *e.g.*, by 1-10%, as, for example, by standard methods of doping the phosphoramidite precursors used in oligonucleotide synthesis with small amounts of contaminating phosphoramidite precursors. Such methods are well known in the art. Also, see Section 7.2.3.

5.4. APPLICATIONS AND USES OF REAGENTS BINDING VINCULIN, DYNEIN, AND GLUTATHIONE S-TRANSFERASE

The reagents binding vinculin, dynein, or glutathione S-transferase of the present invention are useful for in vitro and in vivo applications which heretofore have been typically performed by binding

regions of antibodies. Therefore, the reagents of the present invention may be used as a substitute for an antibody in many of the reactions or assays that the antibody could be used in. For example, the reagents of the present invention could be used in immunoassays known in the art, *e.g.*, those designed to detect or measure the amount of the antigen where the antigen is vinculin, dynein, or glutathione S-transferase. Of course, such immunoassays may have to be suitably modified. For example, many immunoassays make use of a step in which a second antibody, labeled with a radioactive moiety or an enzyme such as alkaline phosphatase, specifically binds to the first antibody. Such a second antibody would not be expected to specifically bind to the reagent of the present invention. However, it would be well within the competence of one of ordinary skill in the art to fabricate another labelling moiety, perhaps a third antibody, that was able to specifically bind to the reagent, or to label the reagent with a detectable marker prior to use.

The immunoassay formats which can be used include but are not limited to competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assays), "sandwich" immunoassays, dot immunoblot assays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, immunohistology assays, protein A immunoassays, immunoaffinity chromatography, and flow dipstick assays to name but a few. For examples of exemplary procedures which can be used in immunoassays, see generally Kricka, 1985, Clinical and Biochemical Analysis 17: 1-15; Armbruster, 1993, Clin. Chem. 39/2: 181-195; Birnbaum et al., 1992, Anal. Biochem. 206: 168-171; Miyai, 1985, Adv. Clin. Chem. 24: 61-110; and references cited therein.

The samples to be assayed in the immunoassays can be any sample that may contain vinculin, dynein, or glutathione S-transferase.

The detectable label to be used in the immunoassays can be any detectable label known in the art. Such labels include radioisotopes, fluorescent dyes, enzymes (for example horseradish peroxidase or alkaline phosphatase), chemiluminescent molecules, metal atoms, or phosphorescent dyes, colored particles, metal and dye colloids.

The discussion that follows is framed in terms of TSARs and TSAR compositions solely for ease of explanation. It will be evident to one of ordinary skill in the art that the uses described for TSARs will be equally applicable to all binding reagents of the present invention.

The TSAR products can be used in any industrial or pharmaceutical application that uses a peptide binding moiety specific for a given ligand. The TSARs can also be intermediates in the production of unifunctional binding peptides that are produced and selected by the method of the invention to have a binding affinity, specificity and avidity for a given ligand. The TSARs and TSAR compositions can also be used to identify promising leads for developing new drug candidates for a variety of therapeutic and/or prophylactic applications. Thus, according to the present invention, TSARs and TSAR compositions are used in a wide variety of applications, including but not limited to, uses in the field of biomedicine, chemistry, catalysis, pharmaceuticals, etc. The applications described below are intended as illustrative examples of the uses of TSARs and compositions comprising the reagent of the binding domain of a TSAR and are in no way intended as a limitation thereon. Other applications will be readily apparent to those of skill in the art and are intended to be encompassed by the present invention.

The TSARs and TSAR compositions are useful in a wide variety of <u>in vivo</u> applications in the fields of biomedicine, bioregulation, and control. In certain of these applications, the TSARs are employed as mimetic replacements for compositions such as enzymes, immunoglobulins, and adhesive proteins.

The invention provides methods of treatment by administration to a subject of an effective amount of a pharmaceutical (therapeutic or diagnostic) composition comprising a binding molecule specific to vinculin, dynein, or glutathione S-transferase. Such a molecule envisioned for therapeutic or diagnostic use is referred to hereinafter as a "Therapeutic" or "Therapeutic of the invention." Such therapeutics are peptides that specifically bind to vinculin, dynein, or glutathione S-transferase *in vivo*, to exert a therapeutic or diagnostic effect. In a preferred aspect, the Therapeutic is substantially purified. The subject is preferably an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human.

Formulations and methods of administration that can be employed are known in the art and can be selected from among those described hereinbelow.

Various delivery systems are known and can be used to administer a Therapeutic of the invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, recombinant cells containing the Therapeutic, receptor-mediated endocytosis (see, *e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262: 4429-4432), etc. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes as well as transdermal and subcutaneous time-release implants. The Therapeutics may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir,

such as an Ommaya reservoir. In a specific embodiment. it may be desirable to utilize liposomes targeted via peptides specific to vinculin, dynein, or glutathione S-transferase.

In a specific embodiment, it may be desirable to administer the Therapeutics of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, *e.g.*, in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous. non-porous, or gelatinous material, including membranes. such as sialastic membranes, or fibers.

The present invention provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a Therapeutic, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The carrier and composition can be sterile. The formulation should suit the mode of administration.

The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol. lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a

local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The Therapeutics of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the Therapeutic of the invention which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of

pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

By virtue of their affinity for vinculin, dynein, or glutathione S-transferase, TSARs or compositions comprising a TSAR binding domain having binding affinity for vinculin, dynein, or glutathione S-transferase or a portion thereof can be used in vivo to deliver a chemically or biologically active moiety to a a site where vinculin, dynein, or glutathione S-transferase is located. The TSARs can also have in vitro a utility similar to monoclonal antibodies or other specific binding molecules for the detection, quantitation, separation or purification of vinculin, dynein, or glutathione S-transferase. In one embodiment, a number of TSARs or the binding domains thereof can be assembled as multimeric units to provide multiple binding domains that have the same specificity and can be fused to another molecule that has a biological or chemical activity.

In the chemical industry, TSARs can be employed for use in separations, purifications, preparative methods, and catalysis.

In the field of diagnostics, TSARs binding vinculin, dynein, or glutathione S-transferase can be used to detect ligands occurring in lymph, blood, urine, feces, saliva, sweat, tears, mucus, or any other physiological liquid or solid. In the area of histology and pathology, TSARs can be used to detect ligands in tissue sections, organ sections, smears, or in other specimens examined macroscopically or microscopically. TSARs can also be used in other diagnostics as replacements for antibodies. The TSARs of the present invention can be used in place of antibodies in enzyme linked immunosorbent assays to detect or quantitate vinculin, dynein, or glutathione S-transferase.

The methods of the present invention may be used to identify TSARs that are inhibitors for enzymes such as glutathione S-transferase. Once a TSAR that binds to an enzyme is identified, that TSAR can be tested for inhibitory activity by adding it to standard assays for the activity of the enzyme. Such TSARs or the binding domains thereof may be formulated into

therapeutic compositions for use in inhibiting the activity of such enzymes *in vivo*. In particular, TSARs binding glutathione S-transferase may be used in chemotherapy against tumors that have an elevated level of glutathione S-transferase activity.

The present invention further provides reagents and compositions that bind to vinculin for altering the mobility or attachability of malignant cells, as well as modulating platelet release and blood clotting. Also provided are methods of identifying a series of TSAR reagents that show progressively increased binding affinity for vinculin.

5.5 SYNTHESIS OF PEPTIDES

5.5.1 PROCEDURE FOR SOLID PHASE SYNTHESIS

Peptides may be prepared by methods that are known in the art. For example, in brief, solid phase peptide synthesis consists of coupling the carboxyl group of the C-terminal amino acid to a resin and successively adding N-alpha protected amino acids. The protecting groups may be any known in the art. Before each new amino acid is added to the growing chain, the protecting group of the previous amino acid added to the chain is removed. The coupling of amino acids to appropriate resins is described by Rivier et al., U.S. Patent No. 4,244,946. Such solid phase syntheses have been described, for example, by Merrifield, 1964, J. Am. Chem. Soc. 85: 2149; Vale et al., 1981, Science 213: 1394-1397; Marki et al., 1981, J. Am. Chem. Soc. 103: 3178 and in U.S. Patent Nos. 4,305,872 and 4,316,891. In a preferred aspect, an automated peptide synthesizer is employed.

By way of example but not limitation, peptides can be synthesized on an Applied Biosystems Inc. ("ABI") model 431A automated peptide synthesizer using the "Fastmoc" synthesis protocol supplied by ABI, which uses 2-(1H-Benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate ("HBTU") (R. Knorr et al., 1989, Tet. Lett., 30: 1927)

as coupling agent. Syntheses can be carried out on 0.25 mmol of commercially available 4-(2',4'-dimethoxyphenyl-(9-fluorenyl-methoxycarbonyl)-aminomethyl)-phenoxy polystyrene resin ("Rink resin" from Advanced ChemTech) (H. Rink, 1987, Tet. Lett. 28: 3787). Fmoc amino acids (1 mmol) are coupled according to the Fastmoc protocol. The following side chain protected Fmoc amino acid derivatives are used: FmocArg(Pmc)OH; FmocAsn(Mbh)OH; FmocAsp(^{t}Bu)OH; FmocCys(Acm)OH; FmocGlu(^{t}Bu)OH; FmocGln(Mbh)OH; FmocHis(Tr)OH; FmocLys(Boc)OH; FmocSer(^{t}Bu)OH; FmocThr(^{t}Bu)OH; FmocTyr(^{t}Bu)OH. [Abbreviations: Acm, acetamidomethyl; Boc, tert-butoxycarbonyl; ^{t}Bu, tert-butyl; Fmoc, 9-fluorenylmethoxycarbonyl; Mbh, 4,4'-dimethoxybenzhydryl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Tr, trityl].

Synthesis is carried out using N-methylpyrrolidone (NMP) as solvent, with HBTU dissolved in N,N-dimethylformamide (DMF). Deprotection of the Fmoc group is effected using ca. 20% piperidine in NMP. At the end of each synthesis the amount of peptide present is assayed by ultraviolet spectroscopy. A sample of dry peptide resin (ca. 3-10 mg) is weighed, then 20% piperidine in DMA (10 mL) is added. After 30 min sonication, the UV (ultraviolet) absorbance of the dibenzofulvene-piperidine adduct (formed by cleavage of the N-terminal Fmoc group) is recorded at 301 nm. Peptide substitution (in mmol g^{-1}) can be calculated according to the equation:

$$\text{substitution} = \frac{A \times v}{7800 \times w} \times 1000$$

where A is the absorbance at 301 nm, v is the volume of 20% piperidine in DMA (in mL), 7800 is the extinction coefficient (in $mol^{-1}dm^3cm^{-1}$) of the dibenzofulvene-piperidine adduct, and w is the weight of the peptide-resin sample (in mg).

Finally, the N-terminal Fmoc group is cleaved using 20% piperidine in DMA, then acetylated using acetic anhydride and pyridine in DMA. The peptide resin is thoroughly washed with DMA, CH_2Cl_2 and finally diethyl ether.

5.5.2 CLEAVAGE AND DEPROTECTION

By way of example but not limitation, cleavage and deprotection can be carried out as follows: The air-dried peptide resin is treated with ethylmethyl-sulfide (EtSMe), ethanedithiol (EDT), and thioanisole (PhSMe) for approximately 20 min. prior to addition of 95% aqueous trifluoracetic acid (TFA). A total volume of ca. 50 mL of these reagents are used per gram of peptide-resin. The following ratio is used: TFA : EtSMe : EDT : PhSme (10 : 0.5 : 0.5 : 0.5). The mixture is stirred for 3 h at room temperature under an atmosphere of N_2. The mixture is filtered and the resin washed with TFA (2 x 3 mL). The combined filtrate is evaporated *in vacuo*, and anhydrous diethyl ether added to the yellow/orange residue. The resulting white precipitate is isolated by filtration. See King et al., 1990, Int. J. Peptide Protein Res. 36: 255-266 regarding various cleavage methods.

5.5.3 PURIFICATION OF THE PEPTIDES

Purification of the synthesized peptides can be carried out by standard methods including chromatography (*e.g.*, ion exchange, affinity, and sizing column chromatography, high performance liquid chromatography (HPLC)), centrifugation, differential solubility, or by any other standard technique.

5.5.4 CONJUGATION OF PEPTIDES TO OTHER MOLECULES

The peptides of the present invention may be linked, *i.e.* conjugated, to other molecules (*e.g.*, a detectable label, a molecule facilitating adsorption to a solid substratum, or a toxin, according to various embodiments

of the invention) by methods that are well known in the art. Such methods may be covalent or non-covalent, *i.e.* the other molecule may be linked to the peptide by a covalent bond or by some other method such as, for example, ionic bonds, hydrogen bonds, van der Waals forces, a biotin-streptavidin link, etc. The peptides of the present invention may be conjugated or linked to a second molecule that may be an amino acid, a peptide, a protein, a nucleic acid, a nucleoside, a glycosidic residue, a label, a drug, and a small molecule.

Methods of conjugation include the use of homobifunctional and heterobifunctional cross-linking molecules. The homobifunctional molecules have at least two reactive functional groups, which are the same. The reactive functional groups on a homobifunctional molecule include, for example, aldehyde groups and active ester groups. Homobifunctional molecules having aldehyde groups include, for example, glutaraldehyde and subaraldehyde. The use of glutaraldehyde as a cross-linking agent was disclosed by Poznansky et al., 1984, Science 223: 1304-1306.

Homobifunctional molecules having at least two active ester units include esters of dicarboxylic acids and N-hydroxysuccinimide. Some examples of such N-succinimidyl esters include disuccinimidyl suberate and dithio-bis-(succinimidyl propionate), and their soluble bis-sulfonic acid and bis-sulfonate salts such as their sodium and potassium salts. These homobifunctional reagents are available from Pierce, Rockford, Illinois.

The heterobifunctional molecules have at least two different reactive groups. Some examples of heterobifunctional reagents containing reactive disulfide bonds include N-succinimidyl 3-(2-pyridyl-dithio)propionate (Carlsson et al., 1978, Biochem J. 173: 723-737), sodium S-4-succinimidyloxycarbonyl-alpha-methylbenzylthiosulfate, and 4-succinimidyloxycarbonyl-alpha-methyl-(2-pyridyldithio)toluene. N-succinimidyl 3-(2-pyridyldithio)propionate is preferred. Some examples of heterobifunctional reagents comprising reactive groups having a double bond that reacts with a thiol group include succinimidyl

4-(N-maleimidomethyl)cyclohexahe-1-carboxylate and succinimidyl m-maleimidobenzoate.

Other heterobifunctional molecules include succinimidyl 3-(maleimido)propionate, sulfosuccinimidyl 4-(p-maleimido-phenyl)butyrate, sulfosuccinimidyl 4-(N-maleimidomethyl-cyclohexane)-1-carboxylate, maleimidobenzoyl-N-hydroxy-succinimide ester. The sodium sulfonate salt of succinimidyl m-maleimidobenzoate is preferred. Many of the above-mentioned heterobifunctional reagents and their sulfonate salts are available from Pierce.

Additional information regarding how to make and use these as well as other polyfunctional reagents may be obtained from the following publications or others available in the art:

Carlsson et al., 1978, Biochem. J. 173: 723-737.

Cumber et al., 1985, Methods in Enzymology 112: 207-224.

Jue et al., 1978, Biochem 17: 5399-5405.

Sun et al., 1974, Biochem. 13: 2334-2340.

Blattler et al., 1985, Biochem. 24: 1517-152.

Liu et al., 1979, Biochem. 18: 690-697.

Youle and Neville, 1980, Proc. Natl. Acad. Sci. USA 77: 5483-5486.

Lerner et al., 1981, Proc. Natl. Acad. Sci. USA 78: 3403-3407.

Jung and Moroi, 1983, Biochem. Biophys. Acta 761: 162.

Caulfield et al., 1984, Biochem. 81: 7772-7776.

Staros, 1982, Biochem. 21: 3950-3955.

Yoshitake et al., 1979, Eur. J. Biochem. 101: 395-399.

Yoshitake et al., 1982, J. Biochem. 92: 1413-1424.

Pilch and Czech, 1979, J. Biol. Chem. 254: 3375-3381.

Novick et al., 1987, J. Biol. Chem. 262: 8483-8487.

Lomant and Fairbanks, 1976, J. Mol. Biol. 104: 243-261.

Hamada and Tsuruo, 1987, Anal. Biochem. 160: 483-488.

Hashida et al., 1984, J. Applied Biochem. 6:56-63.

Additionally, methods of cross-linking are reviewed by Means and Feeney, 1990, Bioconjugate Chem. 1:2-12.

5.5.4.1 BIOTINYLATION OF PEPTIDES

Methods of biotinylating peptides are well known in the art. Any convenient method may be employed in the practice of the invention. For example, the following procedure may be used:

(1) dissolve 10 mg of peptide in 100 μL of 0.1 % acetic acid;

(2) add 900 μL of PBS;

(3) add 3.3 mg of biotin-LC-NHS (Pierce, Rockford, IL);

(4) incubate for 30 minutes at room temperature;

(5) purify over a Superose 12 column (Pharmacia, Piscataway, NJ).

5.5.4.2 CYCLIZATION OF PEPTIDES

Methods of cyclizing peptides with two cysteine residues are well known in the art. Such methods include oxidation to disulfide bonds by air, using iodine or ferricyanide, or other methods. See Stuart, J.M. and Young, J.B., 1984, Solid Phase Peptide Synthesis, 2nd ed., Pierce, Rockford, ILL.

The following examples are presented for purposes of illustration only and are not intended to limit the scope of the invention in any way.

6. EXAMPLE: PREPARATION OF TSAR LIBRARIES

TSAR libraries were prepared as set forth below.

6.1. PREPARATION OF THE TSAR-9 LIBRARY

6.1.1. SYNTHESIS AND ASSEMBLY OF OLIGONUCLEOTIDES

Synthesis and assembly of the oligonucleotides of the TSAR-9 library are described in Kay et al., 1993, Gene 128: 59-65.

6.1.2. CONSTRUCTION OF VECTORS

The construction of the M13 derived phage vectors useful for expressing a TSAR library, *e.g.*, TSAR-9, has been described (Fowlkes et al., 1992, BioTechniques, 13:422-427). To express the TSAR-9 library, an M13 derived vector, m663, was constructed as described in Fowlkes. Figure 1 of Fowlkes illustrates the m663 vector which contains the pIII gene having a c-myc-epitope, *i.e.*, as a stuffer fragment, introduced at the mature N-terminal end, flanked by Xho I and Xba I restriction sites.

6.1.3. EXPRESSION OF THE TSAR-9 LIBRARY

The synthesized oligonucleotides were ligated to Xho I and Xba I double-digested m663 RF DNA containing the pIII gene (Fowlkes, supra) by incubation with ligase overnight at 12°C. The ligated DNA was then introduced into E. coli by electroporation. Details of this process can be found in Kay et al., 1993, Gene 128: 59-65.

6.2. PREPARATION OF TSAR-12 LIBRARY

Figure 1 shows the formula for the synthetic oligonucleotides and the assembly scheme used in the construction of the TSAR-12 library. As shown in Figure 1, the TSAR-12 library was prepared in substantially the same manner as the TSAR-9 library described in Kay et al., 1993, Gene 128: 59-65 with the following exceptions: (1) each of the variant non-predicted oligonucleotide sequences, was 30 nucleotides in length, rather than 54 nucleotides; (2) the restriction sites included at the 5′ termini of the variant, non-predicted sequences were Sal I and Spe I, rather than Xho I and Xba I; and (3) the invariant sequence at the 3′ termini to aid annealing of the two strands was GCGGTG rather than CCAGGT (5′ to 3′).

After synthesis including numerous rounds of annealing and chain extension in the presence of dNTP's and Taq DNA polymerase, and purification, the synthetic double stranded oligonucleotide fragments were digested with Sal I and Spe I restriction enzymes and ligated with T4 DNA ligase to the nucleotide sequence encoding the M13 pIII gene contained in the m663 vector to yield a library of TSAR-12 expression vectors. The ligated DNA was then introduced into E. coli (DH5αF′; GIBCO BRL, Gaithersburg, MD) by electroporation. The library of E. coli cells were plated at high density (~400,000 per 150 mm petri plate) for amplification of the recombinant phage. After about 8 hr, the recombinant bacteriophage were recovered by washing for 18 hr with SMG buffer and after the addition of glycerol to 50% were frozen at -80°C.

The TSAR-12 library thus formed had a working titer of ~ 2×10^{11} pfu/ml.

6.3. PREPARATION OF THE R8C LIBRARY

A randon peptide expression library, termed R8C, was prepared as described for the TSAR-9 library described in Kay et al., 1993, Gene 128:

59-65 but with the modifications depicted in Figures 2 and 3. For a description of the m663 vector of Figures 2 and 3, see Fowlkes et al., 1992, BioTechniques, 13:422-427. The oligonucleotide assembly process depicted in Figures 2 and 3 predominantly yields an expressed peptide with a random 8-mer sequence, comprising the following amino acid sequence expressed at the amino terminus of pIII: $SSC(X)_8CGSR$ (SEQ ID NO 19). However, a percentage of the library contains a double insert resulting in expression of a peptide with a random 16-mer sequence (see Figure 3), comprising the following amino acid sequence expressed at the amino terminus of pIII: $SSC(X)_8CGSRST(X)_8TTR$...(SEQ ID NO 13).

7. IDENTIFICATION OF LIGAND BINDING TSARS

In several series of experiments, the TSAR-9, TSAR-12, and R8C libraries described in Section 6 above were screened, according to the present invention, for expressed proteins/peptides having binding specificity for a ligand selected from among vinculin, dynein, or glutathione S-transferase.

7.1. IDENTIFICATION OF TSARs BINDING DYNEIN

In one set of experiments,the TSAR-12 library was screened for expressed proteins/peptides having binding specificity for dynein.

In particular, the TSAR-12 library was screened to identify TSARs binding dynein as follows.

Cytoplasmic dynein was a gift of Dr. Richard Walker (Department of Cell Biology, Duke University); it was purified from chick embryo neural tissue. A small aliquot (50 μl) of cytoplasmic dynein diluted to 1 μg/ml in 100 mM $NaHCO_3$, pH 8.5 was placed into wells of microtiter plates (Corning). The plates were incubated overnight at 4°C, 100 μl of BSA solution (1 mg/ml, in 100 mM $NaHCO_3$, pH 8.5) was added and the plates were incubated at room temperature for 1 hr. The microtiter plates were

emptied and the wells washed carefully with PBS-0.05% Tween 20, using a squeeze bottle. PBS is 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4-$7H_2O$, 1.4 mM KH_2PO_4, pH 7.3. Then 25 μl of TSAR-12 phage solution (~10^{14} pfu/ml) was introduced into each well and the plates were incubated at room temperature for 1-2 hours. The contents were removed and the wells filled carefully with PBS-0.05% Tween 20, using a squeeze bottle. The plates were washed five times to remove unbound phage. The plates were incubated with wash solution for 20 minutes at room temperature to allow bound phage with rapid dissociation constants to be released. The wells were then washed five more times to remove any remaining unbound phage.

The phage bound to the wells were recovered by elution with a pH change. Fifty microliters of 50 mM glycine·HCl (pH 2.2), 100 μg/ml BSA solution were added to washed wells to denature proteins and release bound phage. After 5-10 minutes, the contents were then transferred into clean tubes, and 100 μl 1 M Tris·HCl (pH 7.5) or 1 M NaH_2PO_4 (pH 7) was added to neutralize the pH of the phage sample. The phage were then diluted 10^{-3} to 10^{-6} and aliquots plated with E. coli DH5αF cells to determine the number of plaque forming units of the sample. The titer of the input samples was also determined for comparison (dilutions are generally 10^{-6} to 10^{-9}).

Successful screening experiments have generally entailed 3 rounds of serial screening. Serial screening was conducted in the following manner. First, the library was screened and the recovered phage rescreened immediately. Second, the phage that were recovered after the second round were plate amplified according to Sambrook, Fritsch and Maniatis, 1989, Molecular Cloning: A Laboratory Manual, 2d. ed. Cold Spring Harbor Laboratory Press. The phage were eluted into SMG (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.05% gelatin) by overlaying the plates with ~5ml of SMG and incubating the plates at 4°C overnight. Third, a small aliquot was then taken from the plate and rescreened. The recovered

phage were then plated at a low density to yield isolated plaques for individual analysis.

The individual plaques were picked with a toothpick and used to inoculate cultures of E. coli F' cells in 2xYT. After overnight culture at 37°C, the cultures were spun down by centrifugation. The liquid supernatant was then transferred to a clean tube and served as the phage stock. Generally, it has a titer of 10^{12} pfu/ml that is stable at 4°C. Binding to cytoplasmic dynein was confirmed for four isolates. The inserted oligonucleotides of the four phage were determined by DNA sequencing; this technique showed that the four isolates were identical and likely represented siblings. The amino acid sequence was deduced with a MacVector™ computer program.

The amino acid sequence of the binding domain of the TSARs encoded by the dynein binding phage is shown in Table 1.

Table 1

TSARs BINDING DYNEIN

Amino Acid Sequence[1]	Name	SEQ ID NO
WVMLGYCAKAGGAHRDRMRTAIC	Dyn-12.1	20

[1] The non-variable amino acids at the NH_2 and COOH terminal residues are not shown.

Based on the results obtained, the dynein-binding TSAR and peptides comprising portions of such TSAR, such as SEQ ID NO 20, for example, should be useful for modulating nerve cell activity, the ciliary action of respiratory cells in patients with cystic fibrosis, sperm cell motility, and to inhibit mobility of protozoan parasites, such as Trypanosomes, etc. In addition, the dynein-binding TSARs and TSARs compositions can be used to identify and develop new drug candidates for modulation of various conditions affecting the nervous system, the respiratory tract, as well as therapeutic

and/or prophylactic treatment of a variety of conditions caused by infections microorganisms or parasites. Also, the peptide shown in Table 1, linked to biotin or other labels, is useful in cytohistology for determining the distribution of dynein in cells. The peptide coupled to beads or other solid supports is useful for the affinity purification of dynein from cell extracts.

7.2 IDENTIFICATION OF TSARS BINDING VINCULIN

In another series of experiments, the TSAR-12 library was screened for expressed proteins/peptides having binding specificity for vinculin.

In particular, the TSAR-12 library was screened substantially as described above in Section 7.1, except that vinculin (at 5 μg/ml in 100 mM $NaHCO_3$, pH 8.5, 100 μl per well) was immobilized in the microtiter wells instead of dynein. Vinculin was isolated from chicken gizzards by standard methods well known in the art.

After 3 rounds of serial screening, as described above, the recovered vinculin binding phage were plated at low density to yield isolated plaques for individual analysis. Binding to vinculin was confirmed for four isolates.

The individual plaques were picked with a toothpick and used to inoculate cultures of E. coli F′ cells in 2xYT. After overnight culture at 37°C, the cultures were then spun down by centrifugation. The liquid supernatant was then transferred to a clean tube and served as the phage stock. Generally, it has a titer of 10^{12} pfu/ml that is stable at 4°C. The inserted oligonucleotides of the four phage were determined by DNA sequencing which showed that the four isolates were identical and likely represented sibling clones. The amino acid sequence was deduced with a MacVector™ computer program.

The amino acid sequence of the binding domain of the TSARs encoded by the vinculin binding phage is shown in Table 2.

Table 2

TSARs BINDING VINCULIN

Amino Acid Sequence[1]	Name	SEQ ID NO
GGFDDVYDWARGVSSALTTLVA	Vin 12.1	21

[1] The non-variable amino acids at the NH_2 and COOH terminal residues are not shown.

Based on the results obtained, the vinculin-binding TSAR and peptides comprising portion of such TSAR. such as SEQ ID NO 21, for example, should be useful for modulating how cells move and/or bind to sites in the body. For example, vinculin-binding TSARs, or portions thereof, may alter the mobility or attachability of malignant cells, perhaps preventing or inhibiting metastasis. Vinculin-binding TSARs, or portions thereof, may also modulate platelet release and blood clotting. Also, the peptide shown in Table 2, linked to biotin or other labels, is useful in cytohistology for determining the distribution of vinculin in cells. The peptide coupled to beads or other solid supports is useful for the affinity purification of vinculin from cell extracts.

7.2.1. USE OF VINCULIN BINDING TSARS IN ELISA

Vinculin was purified from 50 g of chicken gizzard as described previously (Feramisco et al., 1980, J. Biol. Chem 255: 1194-1199). Three ml fractions were collected from the anion-exchange column. 0.2 μg of vinculin/well was absorbed to 96-well enzyme linked immunosorbent assay (ELISA) plates in 0.1 M sodium bicarbonate for one hour at 37°C. The wells were then blocked with 1% BSA in 0.1 M sodium bicarbonate. Ten μl phage supernatant was added to each well containing 200 μl wash buffer (1X PBS,

0.1% Tween-20, 0.1% BSA). After one hour incubation, the wells were washed repeatedly, then the number of remaining bound phage particles determined using a horseradish-peroxidase coupled an anti-M13 antibody (Pharmacia, Piscataway, NJ) following the manufacturer's protocol. The sequences of the phage-displayed peptides were deduced by sequencing (Sequenase version 2.0, United States Biochemical Corp., Cleveland, OH) both DNA strands of the appropriate region in the viral genome.

Figure 4 shows the results of ELISAs using Vin 12.1 and the negative control phage Nonvin 1.1, a random isolate from the R8C library. Vin 12.1 specifically recognizes vinculin from the column fractions that, judging by SDS-PAGE, contain the highest levels of vinculin.

7.2.2. USE OF VINCULIN BINDING TSAR IN WESTERN BLOT

A peptide, pVin 12.1, corresponding to the amino acid sequence of the insert of Vin 12.1 (SEQ ID NO 21) was prepared using standard techniques of peptide synthesis. pVin 12.1 was biotinylated, again according to standard techniques well known in the art.

Protein samples from the anion exchange column purification of vinculin were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% polyacrylamide gel. The samples were transferred to Immobilon nitrocellulose membranes using standard western blotting techniques and the membrane was blocked with polyvinyl alcohol. Next, 0.1 μg biotinylated pVin.12-1 was preincubated with 1.0 μg streptavidin alkaline phosphatase in wash buffer for one hour. This mixture was incubated with the membrane for one hour, washed 4 x 15 min in wash buffer and then alkaline phosphatase activity determined by adding p-nitrophenylphosphate and measuring absorbance at 405 nm.

Figure 5 shows the results of this western blot. pVin 12.1 specifically recognizes a 130 kD band representing vinculin. pVin 12.1 non-specifically (as shown below) recognizes a 71 kD band.

A further western blot was done by running out the following samples on an SDS polyacrylamide gel: (1) purified vinculin; (2) a whole cell lysate of chicken embryo fibroblasts isolated from the skin of 10-day chicken embryos (CEF cells); (3) fraction 21 from the column of Figure 5; (4) a GST-SH3 domain fusion protein (see Sparks, A.B. et al., 1994, J. Biol Chem. 269: 23853-23856). A western transfer of the proteins was done and the membrane was probed using streptavidin conjugated pVin 12.1 and pA9. pA9 is a peptide that specifically recognizes the SH3 domain found on many proteins involved in intracellular signaling (see Sparks, A.B. et al., 1994, J. Biol Chem. 269: 23853-23856). The results are shown in Figure 6. It can be seen that pVin 12.1 specifically recognizes the 130 kD vinculin band while pA9 specifically recognizes the GST-SH3 fusion protein. Both peptides recognize the 71 kD band, showing that the recognition of this band by pVin 12.1 is non-specific.

7.2.3. MUTAGENESIS OF VINCULIN BINDING TSAR

Mutagenesis of Vin 12.1 was carried out by deliberately contaminating the precursor reservoirs used to construct the oligonucleotides that were used to make the mutated phage. Oligonucleotides encoding pVin.12-1 (the peptide insert of Vin 12.1) were synthesized in a similar manner as those of the original TSAR-12 library (see above Section 6.2) except that each phosphoramidite base was doped with a total of 5% of each of the other three phosphoramidite bases. A library was then constructed as described above for the TSAR-12 library (Section 6.2).

Random isolates from this library were tested for the ability to bind vinculin by ELISA. Phage with both reduced and with increased binding ability relative to the parent Vin 12.1 phage were identified. The amino acid sequences of the inserts of these phage were determined. Table 3 shows the amino acid sequences of the inserts of the mutated phage that bound vinculin at least 10-fold less than Vin 12.1. The sequence of the peptide displayed by

Vin 12.1 is shown on the bottom row. Amino acid changes from this sequence are indicated with capital letters. A starred position represents a silent mutation, *i.e.*, a change in the nucleotide sequence of the phage that does not result in a change in the amino acid sequence. Underlined positions indicate residues fixed in the TSAR-12 library. This analysis indicates a significant portion of the peptide is involved with binding and single amino acid changes can significantly reduce binding.

TABLE 3

Non-Binders

	SEQ ID NO
. . . C	22
. . . . . C E	23
. . . . . C . . . *	24
. . . . . E . . . . * . . .	25
. . . . . A . . . . *	26
. . . D . . . . Q	27
. . . . . Y E . . . . K	28
. . . . L Y . . . . . I . . . . . *	29
. . . . . S . . I	30
. . . E V . . . . . R . . . P . . . *	31
. . . . . H	32
. . . . . P . P . *	33
. . . . . D . . . . . I . -	34
. . . . . * . P	35
. . . * . . . . . S . V	36
. . . . . * . . . . . V	37
. . . . . V	38
. . . . . * . . . . . V	39
. . * . . . . . T	40
. . . . . P	41
S T G G F D D V Y D W A R G V S S A L T T T L V A T R	42

Table 4 shows the amino acid sequences of the inserts from the phage that bound vinculin more strongly or at least as strongly as Vin 12.1. Table 4 indicates that many changes at the amino terminus are not disruptive

whereas most changes at the carboxy terminus are disruptive. The phage indicated as G to R bound with four fold greater affinity relative to Vin 12.1. Weak indicates three fold weaker relative affinity.

TABLE 4

Binders

	SEQ ID NO
. . . * L . . *	43
. . . . . L . . . F . . . . (weak)	44
. . . . . E	45
. . . . . H	46
. . . R . . . . . A	47
. . . . . V N . * Y	48
. . . . . L . . . . . * . . . . . *	49
. . . . . A . . . . . *	50
. . . S . . . Y . . . . . * . * . . . . A	51

Improved binders

		SEQ ID NO
. . . . . A		52
. . . . . A		53
. . . . . L		54
. . . . . A . . . . . L		55
. . . . * . A . . . . . R . T		56
. . . * * . V L . . . . . E		57
. . . . . R	G to R	58
. . . . . F . . . . R		59
. . . E . . . . F H . . . . . *		60
. . . . . Y . . . . . * . . . . . *		61
S T G G F D D V Y D W A R G V S S A L T T T L V A T R		62

Of the mutated phage with increased binding ability, one, Vin 12.1 G to R, was especially noteworthy in that a single amino acid change improved binding four-fold as compared to the parent Vin 12.1 phage. See Figure 7. The sequence of the insert of Vin 12.1 G to R was GGFDDVYDWARRVSSALTTTLVA (SEQ ID NO 58).

7.2.4. pVIN 12.1 COMPETES WITH TALIN FOR BINDING TO VINCULIN

Figure 8 shows the results of ELISAs which indicate that when pVin 12.1 is added together with talin, pVin 12.1 significantly reduces the ability of talin to bind to vinculin. The same result is not obtained when pVin 12.1 was incubated with either paxillin or α-actinin. That is, pVin 12.1 is not able to reduce the binding of either paxillin or α-actinin to vinculin. This results shows that pVin 12.1 likely binds to the same site on vinculin as does talin.

Figure 9 shows that this inhibition of talin binding by pVin 12.1 is concentration dependent. Figure 9 also shows that an irrelevant peptide, pA9, does not exhibit the ability to compete with talin for binding to vinculin.

Table 5 shows that the amino acid sequence of the peptide insert of Vin 12.1 G to R possesses homology to the carboxy terminus of talin. The indicated region of talin contains a vinculin binding domain. The regions of similarity are indicated in bold underline. Interestingly, the G to R mutation increases the similarity of the peptide to the talin sequence.

TABLE 5

Vin.12-1 G to R

	SEQ ID NO
GGFDDVYDWARRVSSALTTTLVA	58

C-terminal portion of Talin

		SEQ ID NO
1801	QMMTEAVEDL TTTLNEAASA AGVVGGMVDS ITQAINQLDE	
1841	GPMGDPEGSF VDYQTTMVRT AKAIAVTVQE MVTKSNTSPE	
1881	ELGPLANQLT SDYGRLASQA KPAAVAAENE EIGAHIKHRV	
1921	QELGHGCSAL VTKAGALQCS PSDVYTKKEL IECARRVSEK	
1961	VSHVLAALQA GNRGTQACIT AASAVSGIIA DLDTTIMFAT	63

7.2.5. pVIN 12.1, LIKE TALIN, BINDS TO VINCULIN IN SOLUTION ONLY IF THE CARBOXY-TERMINUS OF VINCULIN IS MISSING

The following experiment is a further demonstration of the functional similarities between the vinculin binding peptides of the present invention and talin, highlighting the usefulness of the vinculin binding peptides of the present invention for applications where talin-like binding activity to vinculin is desired.

Under certain conditions, an intramolecular interaction may exist between the amino-terminal head domain and the carboxy-terminal tail domain of vinculin; this interaction may prevent talin from binding to vinculin (Johnson, R.P. and Craig, S.W., 1994, J. Biol. Chem. 269: 12611-12619 (Johnson and Craig)).

Figure 15 demonstrates that pVin 12.1, like talin, is unable to bind to intact vinculin in solution. However, Figure 15 demonstrates that pVin 12.1 is able to bind to vinculin in solution if the carboxy-terminus has been truncated. This result is similar to the results Johnson and Craig obtained with talin. pVin 12.1 may bind to immobilized vinculin, as for example, immobilized in the wells of microtiter plates, because immobilization may expose the talin-binding region of vinculin.

7.3 IDENTIFICATION OF TSARS BINDING GLUTATHIONE S-TRANSFERASE

In another series of experiments, the TSAR-12 and TSAR-9 libraries were screened for expressed proteins/peptides having binding specificity for glutathione S-transferase (GST).

Bacterially expressed recombinant *Schistosoma japiconium* GST protein was purified from bacterial lysates using glutathione agarose 4B (Pharmacia, Piscataway, NJ), according to the manufacturer's instructions. Bound GST was eluted from the glutathione agarose with 10 mM glutathione in PBS. Microtiter wells were coated with GST protein (1-10 μg/well, in 50

mM $NaHCO_3$, pH 8.5) overnight at 4°C and blocked with 1% BSA at room temperature for 1 hr. The wells were washed five times with 200 μl PBS, 0.1% Tween 20, 0.1% BSA.

100 μl TSAR-9 or TSAR-12 phage in PBS, 0.1% Tween 20, 0.1% BSA were added to a well containing immobilized GST protein and allowed to incubate at room temperature for 2 hours. The wells were then washed 5 times. Bound phage were eluted with 100 μl 50 mM glycine (pH 2.2), transferred to a new well, and neutralized with 100 μl of 200 mM NaH_2PO_4 (pH 7.0). Recovered phage were used to infect 1 x 10^9 DH5α F′ E. coli cells in 20 ml 2xYT; the infected cells were grown overnight to result in a 1000 to 10,000-fold amplification of the recovered phage titer. Amplified phage were panned twice more as described above, with the exception of the amplification step. Binding phage recovered after the third round of panning were plated on a lawn of DH5α F′ E. coli cells at a low density (200 PFU per 25 cm^2 plate) to yield isolated plaques for individual analysis.

The individual plaques were picked with a toothpick and used to inoculate cultures of E. coli F′ cells in 2xYT. After overnight culture at 37°C, the cultures were then spun down by centrifugation. The liquid supernatant was transferred to a clean tube and served as the phage stock. Generally, it has a titer of 10^{12} pfu/ml that is stable at 4°C. DNA sequencing of the GST-binding isolates revealed four types of recombinants; many of the phage turned out to be sibling clones. The amino acid sequence of the binding domains of the TSAR phage was deduced with MacVector™ sequence analysis software.

The amino acid sequences of the binding domain of the TSARs encoded by the GST binding phage are shown in Table 6.

Table 6

TSARs BINDING GLUTATHIONE S-TRANSFERASE

Amino Acid Sequence[1]	Name	SEQ ID NO
DVWDFGDHFGGGAMWDLSGYLPA	GST 12.1	64
VERNPWGMGFGGDSSFDDFSAIL	GST 12.2	65
RGSDHFFGGPWDRTSRLSPGLCFKDDGYNAACASTQNT	GST 9.1	66
WFWDDGWLSADAPGGLNSPGSCGVPWSNGSRHRPCTGL	GST 9.2	67

[1] The non-variable amino acids at the NH_2 and COOH terminal residues are not shown.

The GST binding phage were evaluated for the ability to bind GST in the presence or absence of free glutathione, *i.e.*, enzyme substrate. More particularly, the GST binding phage (50 μl of phage stock solution) was added to microtiter wells containing immobilized GST. BSA was used to block non-specific binding. 10 mM glutathione was added to the wells to provide conditions in which glutathione was ether present or absent. The GST binding TSARs did not bind as well in the presence of glutathione. Thus, the enzyme substrate appeared to compete for binding of the GST binding TSARs to GST. These results suggest that the GST binding TSARs bind at, or near, the GST enzyme's glutathione-binding pocket.

Based on the results obtained, the GST-binding TSARs and portions of such TSARs, such as SEQ ID NOs 64-67, for example, should be useful for affinity purification of GST.

7.3.1. ADDITIONAL TSARS BINDING GLUTATHIONE S-TRANSFERASE

In addition to the four GST binding phage from the TSAR-9 and TSAR-12 libraries discussed above, thirteen GST binding phage were

isolated from the R8C library using the screening methods described above. Thus, a total of seventeen GST binding phage were isolated. The amino acid sequences of the inserts of these seventeen GST binding phage are shown in Table 7.

Table 7

GROUP 1		SEQ ID NO
R8C GST 5	CWSEWDGNEC	68
R8C GST 6	CGQWADDGYC	69
R8C GST 15	CEQWDGYGAC	70
R8C GST 16	CWPFWDGSTC	71
R8C GST 19	CMIWPDGEEC	72
R8C GST 23	CESQWDGYDC	73
R8C GST 24	CQQWKEDGWC	74
R8C GST 34	CLYQWDGYEC	75
GST 9.1		
SSRGSDHFFGGPWDRTSRLSPGLCFKDDGYNAACASTQNTSR		76
GST 9.2		
SSWFWDDGWLSADAPGGLNSPGSCGVPWSNGSRHRPCTGLSR		77
GROUP 2		
R8C GST 21	CMGDNLGDDC	78
R8C GST 26	CMGDSLGQSC	79
R8C GST 33	CMDDDLGKGC	80
R8C GST 37	CMGENLGWSC	81
R8C GST 43	CLGESLGWMC	82
GROUP 3		
GST 12.1		
STDVWDFGDHFGGGAMWDLSGYLPATR		83
GST 12.2		
STVERNPWGMGFGGDSSFDDFSAILTR		84

* Bold residues are constraints of the library. Underlined residues are mentioned in the text as of interest.

The seventeen sequences are arranged in three groups. Phage in group 1 contain the sequence DG and share similarities in the amino acids that flank the DG sequence. Phage in Group 2 have the sequence GD or a closly related sequence but do not share the flanking sequences of Group 1. Group 3 contains two phage from the TSAR-12 library which share some of the characteristics of the phage from Group 1 and some of the characteristics

of the phage from Group 2 but also possess features which argue for separate classification.

It is interesting to note several features of the sequences in Table 7. 10 of the 13 R8C clones contain the sequence DG or its inversion, GD. The other three clones (33,37,43) have slight variations of these sequences. The residues flanking the DG sequence in the Group 1 phage seem to follow a pattern. Most often there is an acidic residue (D or E) on one side of the DG; aromatic amino acids (Y, W, F) are often on the other side. This is true in many of the class one R8C phage clones and also in the GST 9.1 clone which shares the double cysteines motif. This pattern is also present in the GST 12.1 clone of Group 3 (which has the GD motif rather than the DG motif of Group 1) as shown by the underlined sequence in Table 7.

Another consensus sequence is evident in the Group 2 phage. LG is perfectly conserved while other sequences (residues one, two, and three after the left cysteine) are almost perfectly conserved. If these sequences were inverted, they would all contain the DG sequence or else a slight modification. However, the surrounding aromatic amino acids that are present in Group 1 are absent in the phage of Group 2. GST 12.2 contains the GD sequence like the phage of Group 2 and contains several surrounding aromatic amino acids like the phage of Group 1. Therefore, it does not seem to fit into either Group 1 or Group 2 and has been placed in group three with the other TSAR-12 clone, GST 12.2. Like the phage of Group 2, GST 12.2 contains the GD sequence without immediately surrounding aromatic residues. However, GST 12.2 lacks the other consensus sequences of the phage of Group 2. Thus, it has been put in Group 3.

Experiments were done to quantify the binding to GST of the various phage clones obtained from the three libraries. In brief, equal amounts of phage supernatant of known titer representing the seventeen clones were added to microtiter wells coated with GST. The phage were allowed to

bind, the wells were washed to remove non-binding phage, the binding phage were eluted, and the percentage of added phage that bound to the wells was determined.

In more detail, the binding assays were done as follows. 3 μg of GST was added to the wells of a microtiter plate which already contained 100 μl of 100 mM $NaHCO_3$. The plate was incubated at room temperature for one hour. All the wells were blocked with 100 μl of 0.5% BSA in 100 mM $NaHCO_3$. The plate was further incubated at room temperature for one hour and then was washed four times with a wash solution consisting of 1XPBS, 0.1% BSA, 0.1% Tween 20. 120 μl of wash solution, followed by 30 μl of phage, was added to each well. For the experiments involving DTT, the wash solution and DTT were combined to make a solution with a final concentration of 5 mM DTT. This solution was added to the well. Phage were added last. For the GSH inhibition experiment, the wash and a solution of 10 mM glutathione and 50 mM Tris, pH 8 were combined to make a solution with a final concentration of 1 mM GSH. This solution was added to the wells first, with phage following. The plates in all variations of the experiment were then incubated at room temperature for one to two hours and were subsequently washed five times in the manner stated before. In the DTT experiment, there was an additional modification: For the DTT wells, the first wash was done in 5 mM DTT diluted in wash solution. 100 μl of 200 mM glycine-HCl, pH 2 was added to each well after washing to elute the bound phage from the GST. This liquid was transferred to a round bottom microtiter plate containing 100 μl 1M $NaHPO_4$ in each well in order to neutralize the solution. 10 fold dilutions were done in 180 μl 1XPBS. The dilutions were then plated using a pronger on 2XYT agar plates and were left to incubate overnight inverted at 37°C. Percent recovery was calculated for each clone by analyzing the platings and using the formula: phage binding after assay/ phage input x 100. Initial phage titers were known.

The results of these experiments are shown in Table 8. It is important to note that in Table 8, under the column labelled "binding (% recovery)", a smaller value indicates stronger binding. For the columns labelled "% binding in glutathione" or "% binding in 5 mM DTT", however, smaller values indicate decreased binding. It is evident from Table 8 that there is a range of % recovery values over all the phage clones and also within the different groups.

TABLE 8

clone	binding (% recovery)	% binding in glutathione	% binding in 5 mM DTT
R8C.GST.5	6.2×10^{-2}	<1%	<1%
6	1.1×10^{-2}	1.3%	2.2%
15	1.4×10^{-2}	<1%	<1%
16	2.0×10^{-1}	<1%	<1%
19	8.5×10^{-2}	<1%	<1%
23	1.1	<1%	<1%
24	1.3×10^{-2}	<1%	<1%
34	1.0	<1%	<1%
21	1.5	<1%	<1%
26	5.2×10^{-2}	<1%	2.6%
33	5.1×10^{-1}	<1%	<1%
37	2.2×10^{-1}	<1%	<1%
43	7.8×10^{-1}	<1%	<1%
T12.11.1	9.3×10^{-1}	<1%	6.2%
T12.18.2	7.0×10^{-2}	1.2%	14.5%
T9.101	1.0×10^{-1}	<1%	<1%
T9.102	1.4	<1%	16.4%

Table 8 also shows the results of the same experiment when 5 mM DTT is added prior to the phage. DTT has a dramatic effect on the R8C binders; it decreases binding 1500 fold in one case. The rationale for this experiment stems from the idea that cyclization is important for binding. When the cyclization is gone due to the reduction of the disulfide bond by DTT, loss of binding follows. The circular structure created by the disulfide bond is a more rigid structure than a simply linear sequence. This may be a reason there were more binders recovered from the R8C library than the TSAR-12 and TSAR-9 libraries. Also, the TSAR-12 binders showed very little decrease in percent binding (16 and 6.9 fold, respectively) in the presence of DTT. This is due to the fact that there are no disulfide bonds, thus no cyclization, in these two binders. The slight decrease observed is probably non-specific. There is a note of interest for the TSAR-9 clones. GST 9.1 contains two cysteines separated by nine amino acids, thereby making it very similar to the R8C library constraints: two cysteines separated by eight amino acids. Thus, it is very likely that there is a disulfide bond and consequently a circular structure in this clone. This is supported by the 382 fold decrease in binding observed for GST 9.1 when 5 mM DTT was added to the microtiter plate. The GST 12.2 clone contains two cysteines also. However, they are further apart (12 amino acids) and when binding was tested in the presence of 1 mM DTT, there was no decrease. Therefore, this shows that cyclization, if present, is not important in the binding of this clone. While discussing cyclization it is also important to consider that while the TSAR-12 clones do not contain cyclization, they are still very effective binders to GST. Thus, they must be interacting with GST in a different way or the amino acids which bind GST in these clones may somehow get into a similar conformation as those in the R8C and TSAR-9 clones.

7.3.2. GLUTATHIONE S-TRANSFERASE BINDING TSARS BIND AT THE ACTIVE SITE

Table 8 contains data for phage binding when glutathione (GSH) was added to the microtiter wells before the phage. The reason for trying to inhibit phage binding with GSH is that GSH, a GST substrate, binds GST at the catalytic site of the enzyme. If binding of the phage is reduced after this occurs, then the phage could have been binding at the catalytic site also. This seems plausible since this crevice in the protein is receptive to binders already and would be an ideal environment for binding to the phage. The results shown in Table 8 support this hypothesis. When 1 mM GSH is added, large decreases in phage binding occur. However, it is important to consider that other interactions may be occurring. The TSAR-12 binders are not inhibited as much as most of the other clones. Therefore it is crucial to ascertain if the sensitivity to inhibition by GSH is due to cyclization in the random peptide sequence. GSH may be acting as a reducing agent by breaking disulfide bonds as DTT does. To prove that this was not the case, calmodulin (CAM) was used as a target protein in an ELISA experiment. Phage from the R8C library that specifically bound CAM were isolated by the methods disclosed in Section 7.1 above, but with CAM rather than dynein as the ligand. These CAM binding phage were added to microtiter wells to which the CAM protein had been previously bound and were assayed for their ability to bind to the CAM in the absence and in the presence of GSH. There was not a substantial decrease in signal in the GSH wells as compared to the wells lacking GSH. Therefore, GSH was not inhibiting these R8C phage by breaking their disulfide bonds.

7.3.3. PEPTIDES CORRESPONDING TO THE INSERTS OF GLUTATHIONE S-TRANSFERASE BINDING TSARS INHIBIT THE ENZYMATIC ACTIVITY OF GLUTATHIONE S-TRANSFERASE

A further experiment was done to explore the idea that the GST binding phage were binding at the active site of GST. To that end, a synthetic

peptide (p18) corresponding to the insert of TSAR-12 phage clone GST 12.1 (SEQ ID NO 83) was synthesized by methods well known in the art. This peptide was used to inhibit the catalysis by GST of the conjugation of GSH and another substrate, 1-chloro-2,4-dinitrobenzene (CDNB). The formation of this conjugate can be measured by a spectrophotometer since the reaction results in a rise in absorbance readings. First, a curve of absorbance versus time was established where time point readings were taken every thirty seconds with GST alone in the reaction mixture. Then, different dilutions of p18 were used with constant amounts of GST to see the effect on the reaction rate.

For the experiments without p18, 684 μl distilled water, 80 μl 10X reaction buffer (1 M potassium phosphate, pH 6.5), 8 μl CDNB (100 μM 1-chloro-2,4-dinitrobenzene in ethanol), and 8 μl of GSH solution (0.154 g reduced glutathione in 5 ml water) were combined in a microcentrifuge tube. The liquid was vortexed and 400 μl of the solution was transferred to a quartz cuvette. The absorbance was read at 340 nm using a spectrophotometer and this solution was used as a blank. The cuvette was emptied. 20 μl of 0.0125 μg/μl dialyzed GST in 1 X PBS (final concentration of 0.022 mM) was added to the remainder of the solution in the microcentrifuge tube. The GST was pipetted directly into the solution and the timer was started immediately. The solution was vortexed quickly and transferred into the cuvette before the first time point. Absorbance readings were taken every thirty seconds.

For the experiments utilizing p18, 644 μl distilled water was used. The other modifications occurred when, before adding the GST, 40 μl of the correct concentration of peptide was pipetted directly into the solution remaining in the microcentrifuge tube and vortexed. The mixture was allowed to sit thirty seconds before the GST was added in the manner detailed before. The experiment was then continued as above.

The results of these experiments are shown as Figure 10. 100 μM, 50 μM. and 10 μM p18 resulted in a 3.25, 2.75, and 1.25 fold decrease,

respectively, in reaction rate at absorbance 0.9. 0.9 is half the maximum absorbance noted in this assay and, although it is not shown on the graph in Figure 10, the 50 μM p18 and 100 μM p18 curves did eventually reach the same maximum value as the other curves. 100 μM p15, a proline rich vinculin fragment, was used as a control peptide. When it was added to the solution mixture instead of p18, the reaction rate rose slightly higher than the rate with GST alone. As another control, p18 and p15, in separate trials, were added to the reaction mixture without GST. In both cases, no change in absorbance was noted over time. Thus, from these results it is evident that the p18 is inhibiting the activity of the enzyme GST in a reversible, competitive manner.

Also, peptides corresponding to the inserts of the R8C phage clones of Table 7 were synthesized by methods well known in the art. Biotinylated forms of these peptides were shown to successfully bind GST in ELISA experiments. 3 μg of GST target protein was bound to the well of a microtiter plate in 100 μl of 100 mM $NaHCO_3$. The plate was then incubated one hour at room temperature. Wells were blocked with 100 μl of 0.5% BSA in 100 mM $NaHCO_3$ and the plate was left at room temperature for one hour. The plate was then washed four times with a wash solution of 1 X PBS, 0.1%BSA, 0.1%Tween 20. 30 μl of anti-target biotinylated peptide was diluted at various concentrations in wash and added to the plate. The plate was then incubated once again at room temperature for one hour. The plate was washed five times with the solution outlined above. Strepavidin-alkaline phosphatase (Sigma, St. Louis, MO, 1 μg/ml) was diluted 1:100 in wash and 1 μg was added to each well. The plate was then incubated at room temperature for one hour and washed four times. 200 μl of pre-combined 100 μl p-nitrophenyl phosphate (pNPP) and 6 ml AP-buffer (50 mM Tris, pH 8.5, 0.1 mM $MgCl_2$, and 0.1 mM $ZnSO_4$) were added to each well. The plate was then read on an ELISA plate reader at a range of 405-450 nm. The results are shown in Figures 12 and 13.

As judged by ELISA, the non-biotinylated forms of these sequences competed for binding to GST with R8C phage clones representing both Group 1 and Group 2 of Table 7. The non-biotinylated forms of these sequences competed the binding of biotinylated peptides corresponding to the insert sequences of those phage. See Figures 12 and 13, respectively.

The ELISAs were done as follows. When no competing peptides were added, 3 μg of GST target protein was bound to the well of a microtiter plate in 100 μl of 100 mM $NaHCO_3$. The plate was then incubated one hour at room temperature. Wells were blocked with 100 μl of 0.5% BSA in 100 mM $NaHCO_3$ and the plate was left at room temperature for one hour. The plate was then washed four times with a wash solution of 1 X PBS, 0.1% BSA, 0.1% Tween 20. 30 μl of anti-target phage were diluted in wash solution and added to the plate. The plate was then incubated once again at room temperature for one hour. The plate was washed five times with the solution outlined above. 180 μl horseradish peroxidase linked anti-M13 phage conjugate (Pharmacia, Piscataway, NJ) diluted 1:5000 in wash buffer was added to the well. The plate was incubated at room temperature for one hour and then washed four times. 200 μl pre-combined 36 ul 30% H_2O_2, 21 ml 1 X 2'-2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium (ABTS) stock solution (made by dissolving 100 mg ABTS in 450 ml of 50 mM citric acid, pH 4.4 plus 0.05% H_2O_2) was added to each well to initiate the color reaction. The plate was read on an ELISA plate reader at 405 nm.

When competing peptides were added, 100 μM non-biotin peptides diluted in wash were added to the plate after the first wash set. Then, 15-30 minutes later, either phage or the biotinylated peptides were added, depending on the nature of the assay.

Figures 11 and 12 show that phage binding was decreased 4-9 fold and peptide binding was decreased similarly. Figure 11 also shows an aspect of the experiment that proves cyclization is important for the binding of the R8C peptides to GST. These peptides were synthesized normally with

cysteines (p-C) flanking the eight amino acid sequences and also with serines replacing the cysteines (p-S). In the graph, it is shown that binding of p23S is lower than that of p23C since p23C is a better competitor of phage binding to GST. There is some binding of p23S to the GST, but it is by no means of the magnitude of binding of the p23C. p23S could be binding if the linear sequence somehow folds in to the correct formation necessary for binding.

Lastly, the competition assays using biotinylated peptides (p-C/S B) also showed that p23C or p23S will compete both p21CB and p23CB, and likewise, p21C or p21S will compete both p21CB and p23CB. This is shown in Figure 12. In the case of p21 competing p23CB, the blocking is less because p21 in general is not binding very well in peptide form. 1 μM p23 gives a better binding signal than 100 μM p21. Perhaps the cyclization was not complete for p21, or perhaps some sequences will not bind as well as peptides as they do as phage.

The same enzymatic inhibitition assays were done with the R8C sequence peptides as was done with the linear peptide, p18. Figure 13 shows that there is a substantial reduction in the reaction rate when 10 μM p23C is added to the reaction mixture. 10uM p23S shows only slight inhibitition. This is parallel with the results from the competition assay results just discussed. Figure 14 shows that 10uM p21C, the poorer binder, has little effect on the rate of the reaction catalyzed by GST. p21C has the same effect as p21S at 10 μM. It is also important to remember that p18 had very little blocking ability at 10uM (see Figure 10). When further experiments are done with different concentrations of peptide, similarly to the p18 results, a dosage dependent effect will be seen.

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed since these embodiments are intended as illustration of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this

invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

A number of references are cited herein, the entire disclosures of which are incorporated herein, in their entirety, by reference.

WHAT IS CLAIMED IS:

1. A method for identifying a protein/polypeptide and/or peptide which binds to a ligand selected from the group consisting of dynein, glutathione S-transferase, and vinculin, said method comprising: screening a library which expresses random proteins/polypeptides and/or peptides, by contacting said library with said ligand under conditions conducive to ligand binding to the proteins/polypeptides and/or peptides expressed by said library and recovering said proteins/polypeptides and/or peptides expressed by said library which bind said ligand.

2. The method of claim 1 where the library is a chemically synthesized library.

3. The method of claim 1 where the library is a biological expression library.

4. A method for identifying a protein, polypeptide and/or peptide which binds to a ligand selected from the group consisting of dynein, glutathione S-transferase, and vinculin, said method comprising: screening a library of recombinant vectors which express a plurality of heterofunctional fusion proteins comprising:

(a) a binding domain encoded by an oligonucleotide comprising unpredictable nucleotides in which the unpredictable nucleotides are arranged in one or more contiguous sequences, wherein the total number of unpredictable nucleotides is greater than or equal to 60 and less than or equal to 600, and

(b) an effector domain encoded by an oligonucleotide sequence encoding a protein or peptide that enhances expression or detection of the binding domain,

by contacting the plurality of heterofunctional fusion proteins with said ligand under conditions conducive to ligand binding; and recovering the fusion proteins which bind said ligand.

5. The method of claim 4, further comprising determining the nucleotide sequence encoding the binding domain of the recovered protein/polypeptide and/or peptide to deduce the amino acid sequence of said binding domain.

6. The method of claim 4, in which the heterofunctional fusion proteins further comprise a linker domain between the binding and effector domains.

7. The method of claim 6, in which the linker domain is selectively susceptible to cleavage by chemical or enzymatic means.

8. The method of claim 4, in which the ligand is dynein.

9. The method of claim 4, in which the ligand is glutathione S-transferase.

10. The method of claim 4, in which the ligand is vinculin.

11. The method of claim 4, in which the plurality of proteins/polypeptides and/or peptides can form semirigid conformational structures.

12. The method of claim 11, further comprising determining the nucleotide sequence encoding the binding domain of a recovered protein/polypeptide and/or peptide which can form a semirigid conformational structure to deduce the amino acid sequence of said binding domain.

13. The method of claim 11, in which each protein/polypeptide and/or peptide comprises at least two invariant cysteine residues, in which each invariant cysteine residue is encoded by nucleotides positioned flanking a contiguous sequence of unpredictable nucleotides, and in which the invariant cysteine residues are separated from each other in the protein by 8 or 9 amino acid residues.

14. The method of claim 4, further comprising determining the nucleotide sequence encoding the binding domain of a recovered heterofunctional fusion protein, to deduce the amino acid sequence of said binding domain; and identifying which parts of the deduced amino acid sequence are relevant for binding of the identified protein, polypeptide and/or peptide to dynein, glutathione S-transferase or vinculin.

15. A method for identifying a protein, polypeptide and/or peptide which inhibits the activity of an enzyme comprising: screening a library which expresses random proteins/polypeptides and/or peptides, by contacting said library with said enzyme under conditions conducive to enzyme binding to said proteins/polypeptides and/or peptides expressed by said library and recovering said proteins/polypeptides and/or peptides expressed by said library which bind said enzyme.

16. A method for identifying a protein, polypeptide and/or peptide which inhibits the activity of an enzyme comprising: screening a

library of recombinant vectors which express a plurality of heterofunctional fusion proteins comprising:

(a) a binding domain encoded by an oligonucleotide comprising unpredictable nucleotides in which the unpredictable nucleotides are arranged in one or more contiguous sequences, wherein the total number of unpredictable nucleotides is greater than or equal to 60 and less than or equal to 600, and

(b) an effector domain encoded by an oligonucleotide sequence encoding a protein or peptide that enhances expression or detection of the binding domain,

by contacting the plurality of heterofunctional fusion proteins with said enzyme under conditions conducive to enzyme binding and recovering the heterofunctional fusion proteins which bind said enzyme.

17. The method of claim 15 where the enzyme is glutathione S-transferase.

18. A protein, polypeptide and/or peptide which binds dynein, glutathione S-transferase, or vinculin recovered according to the method of claim 1.

19. A peptide which binds specifically to dynein, in which the peptide has the amino acid sequence of SEQ ID NO 20.

20. A peptide which binds specifically to glutathione S-transferase, in which the peptide has an amino acid sequence selected from the group consisting of SEQ ID NOs 64-84.

21. A peptide which binds specifically to vinculin, in which the peptide has an amino acid sequence selected from the group consisting of SEQ ID NOs 21 and 43-62.

22. A protein, polypeptide and/or peptide which inhibits the activity of an enzyme identified by the method of claim 15.

23. A protein, polypeptide and/or peptide of claim 22 where the enzyme is glutathione S-transferase.

24. A peptide or fragment thereof which binds specifically to vinculin, in which the peptide includes, positioned anywhere along its sequence, the amino acid sequence of:

$X_1X_2X_3X_4X_5VX_6X_7X_8ARX_9VX_{10}X_{11}ALTX_{12}TLX_{13}A$ (SEQ ID NO 14)

where X_1 is G, R or S;

X_2 is G or E;

X_3 is F or L;

X_4 is D, V, A, H, or E;

X_5 is D, L, Y, or N;

X_6 is Y or F;

X_7 is D, Y, H, or A;

X_8 is W or L;

X_9 is G, R, E, or A;

X_{10} is S or T;

X_{11} is S or A;

X_{12} is T or A; and

X_{13} is V or L.

25. The peptide or fragment of claim 24 in which:

X_1 is G;

X_2 is G or E;

X_3 is F;

X_4 is D, V, or A;

X_5 is D or L;

X_6 is Y or F;

X_7 is D, Y, or H;

X_8 is W;

X_9 is G, R, E, or A;

X_{10} is S or T;

X_{11} is S;

X_{12} is T or A; and

X_{13} is V or L.

26. A peptide which binds specifically to vinculin, in which the peptide has the amino acid sequence:

GGFDDVYDWARRVSSALTTTLVA (SEQ ID NO 58).

27. A peptide which binds specifically to glutathione S-transferase, in which the peptide has the amino acid sequence:

CX_aWDGX_bC (SEQ ID NO 15)

where X is W, S, E, G, Q, A, P, F, M, I, K, L, Y, N, T, or D; and

$a + b = 5$.

28. A peptide which binds specifically to glutathione S-transferase, in which the peptide has the amino acid sequence:

$CX_1X_2X_3X_4LGX_5X_6C$ (SEQ ID NO 16)

where X_1 is M or L;

X_2 is G or D;

X_3 is D or E;

X_4 is N, S, or D;

X_5 is W, K, Q, or D; and

X_6 is D, S, G, or M.

29. A peptide having 5 to 50 amino acid residues including an amino acid sequence of a formula selected from the group consisting of

X_1VX_2,

ARX_3VX_4 (SEQ ID NO 85), and

LTX_5TL (SEQ ID NO 86),

said amino acid sequence being positioned anywhere along the peptide, in which X_1 is D, L, Y, or N; X_2 is Y or F; X_3 is R, G, E, or A; X_4 is S or T; X_5 is T or A; in which said peptide specifically binds vinculin.

30. The peptide of claim 29 in which the amino acid sequence of the peptide contains one of the sequences of said formulas.

31. The peptide of claim 29 in which the amino acid sequence of the peptide contains any two of the sequences of said formulas.

32. The peptide of claim 29 in which the amino acid sequence of the peptide contains all three of the sequences of said formulas.

33. A method of modulating the activity of vinculin or a vinculin binding protein comprising administering to a subject a composition comprising an amount of a peptide of claim 24 or 29 effective to modulate the activity of vinculin or a vinculin binding protein in said subject; and a pharmaceutically acceptable carrier.

34. A conjugate of the peptide of claim 24 or 29 and a second molecule.

35. The conjugate of claim 34 in which said second molecule is selected from the group consisting of an amino acid, a peptide, a protein, a nucleic acid, a nucleoside, a glycosidic residue, a label, a drug, and a small molecule.

36. A method of detecting vinculin in a sample using an immunoassay format comprising: applying the conjugate of claim 35 to said sample under conditions such that the conjugate can bind to vinculin in said sample; and detecting said conjugate bound to vinculin.

37. A kit for determining the presence of vinculin in a sample comprising, in one or more containers, a conjugate of the peptide of claim 35.

38. The kit of claim 37 for determining the presence of vinculin in a sample in which the conjugate is a fusion protein of the peptide of claim 24 or 29.

39. A method of affinity purifying vinculin comprising:

(a) incubating the peptide of claim 24 or 29 immobilized on a solid phase with a fluid containing vinculin under conditions such that vinculin in the fluid can bind to the peptide; and

(b) recovering the vinculin bound to said immobilized peptide.

40. The method of claim 3 in which the library is a biologically expressed, surface displayed random peptide library.

41. The method of claim 40 in which said library is a phage displayed random peptide library.

42. A method of modulating the activity of dynein or a dynein binding protein comprising administering to a subject a composition comprising an amount of the peptide of claim 19 effective to modulate the activity of dynein or a dynein binding protein in said subject; and a pharmaceutically acceptable carrier.

43. A conjugate of the peptide of claim 42 and a second molecule.

44. A conjugate of claim 43 in which said second molecule is selected from the group consisting of an amino acid, a peptide, a protein, a nucleic acid, a nucleoside, a glycosidic residue, a label, a drug, and a small molecule.

45. A method of detecting dynein in a sample comprising: applying a conjugate comprising the peptide of claim 19 and a detectable label to said sample under conditions such that the conjugate can bind to dynein in said sample; and detecting said conjugate bound to dynein.

46. A kit for determining the presence of dynein in a sample comprising, in one or more containers, a conjugate of the peptide of claim 19 and a detectable label.

47. The kit of claim 46 for determining the presence of dynein in a sample in which the conjugate is a fusion protein of the peptide of amino acid sequence SEQ ID NO 20 with a detectable label.

48. A method of affinity purifying dynein comprising:

(a) incubating the peptide of claim 19 immobilized on a solid phase with a fluid containing dynein under conditions such that dynein in the fluid can bind to the peptide; and

(b) recovering the dynein bound to said immobilized peptide.

49. A method of modulating the activity of glutathione S-transferase or a glutathione S-transferase binding protein comprising administering to a subject a composition comprising an amount of the peptide of claim 20, 27, or 28 effective to modulate the activity of glutathione S-transferase or a glutathione S-transferase binding protein in said subject; and a pharmaceutically acceptable carrier.

50. A conjugate of the peptide of claim 20, 27, or 28 and a second molecule.

51. A conjugate of claim 50 in which said second molecule is selected from the group consisting of an amino acid, a peptide, a protein, a nucleic acid, a nucleoside, a glycosidic residue, a label, a drug, and a small molecule.

52. A method of detecting glutathione S-transferase in a sample comprising: applying a conjugate comprising the peptide of claim 20, 27, or 28 and a detectable label to said sample under conditions such that the conjugate can bind to glutathione S-transferase in said sample; and detecting said conjugate bound to glutathione S-transferase.

53. A kit for determining the presence of glutathione S-transferase in a sample comprising, in one or more containers, a conjugate of the peptide of claim 20, 27, or 28 and a detectable label.

54. The kit of claim 53 for determining the presence of glutathione S-transferase in a sample in which the conjugate is a fusion protein of the peptide of claim 20, 27, or 28 with a detectable label.

55. A method of affinity purifying glutathione S-transferase comprising:

(a) incubating a peptide of claim 20, 27, or 28 immobilized on a solid phase with a fluid containing glutathione S-transferase under conditions such that glutathione S-transferase in the fluid can bind to the peptide; and

(c) recovering the glutathione S-transferase bound to said immobilized peptide.

56. A method of detecting or measuring vinculin, dynein, or glutathione S-transferase in a sample, comprising:

(a) contacting said sample with a molecule comprising a peptide capable of specifically binding vinculin, dynein, or glutathione S-transferase under conditions such that specific binding between said molecule and vinculin, dynein, or glutathione S-transferase in the sample can occur; and

(b) detecting or measuring the amount of said binding in which the presence and amount of said binding indicates the presence or amount, respectively, of vinculin, dynein, or glutathione S-transferase in the sample;

in which said peptide is identified by the method of claim 1.

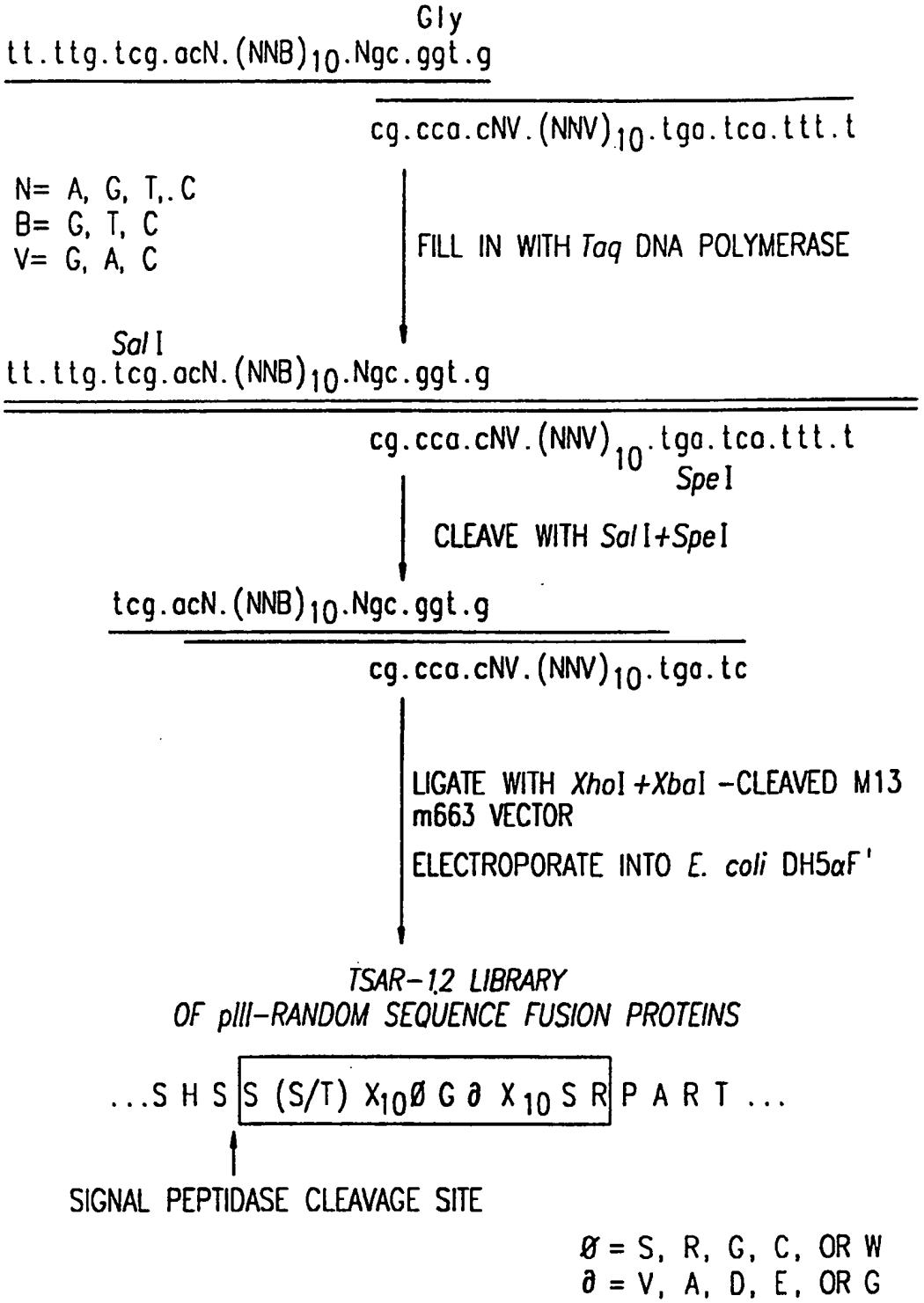
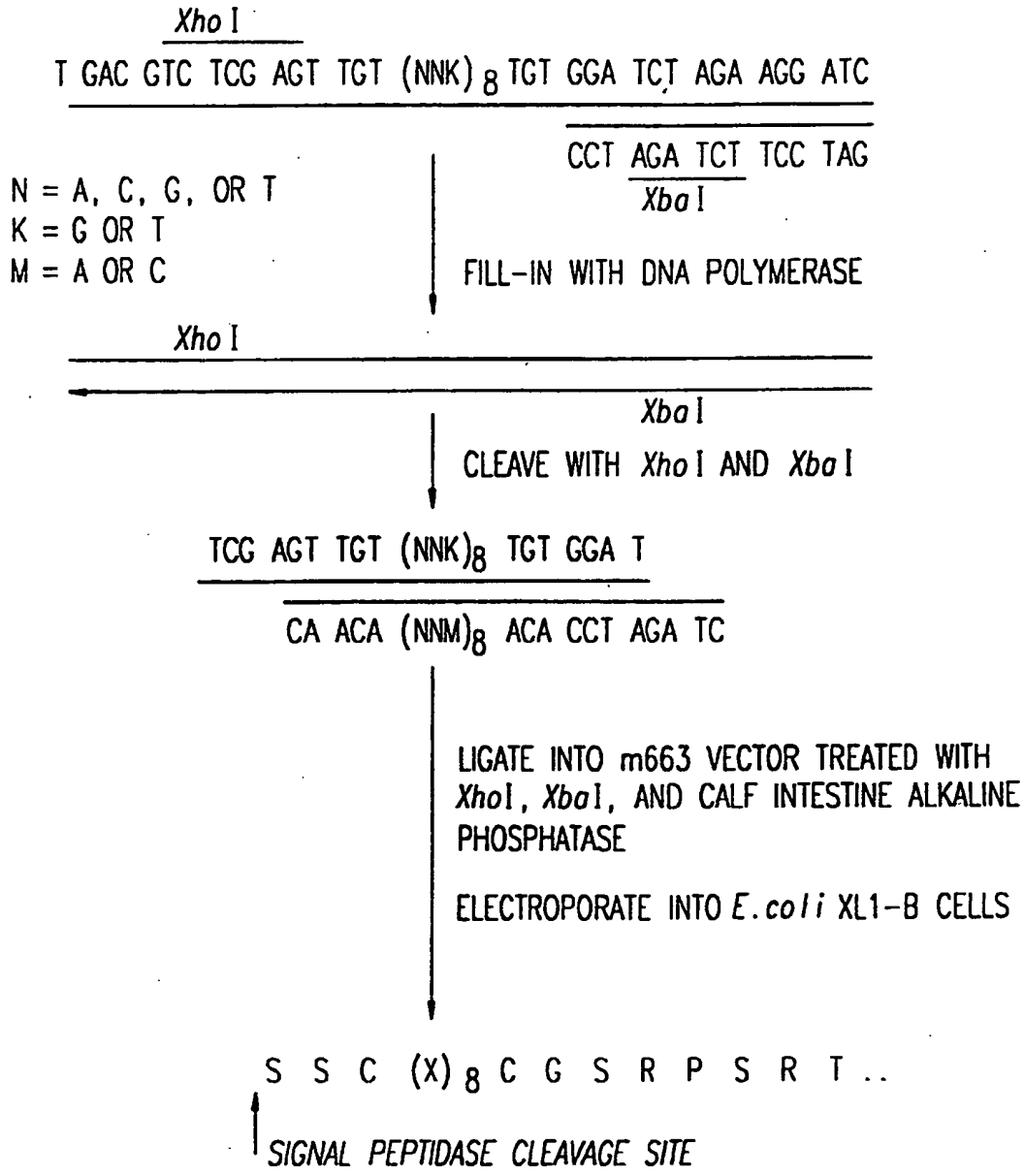
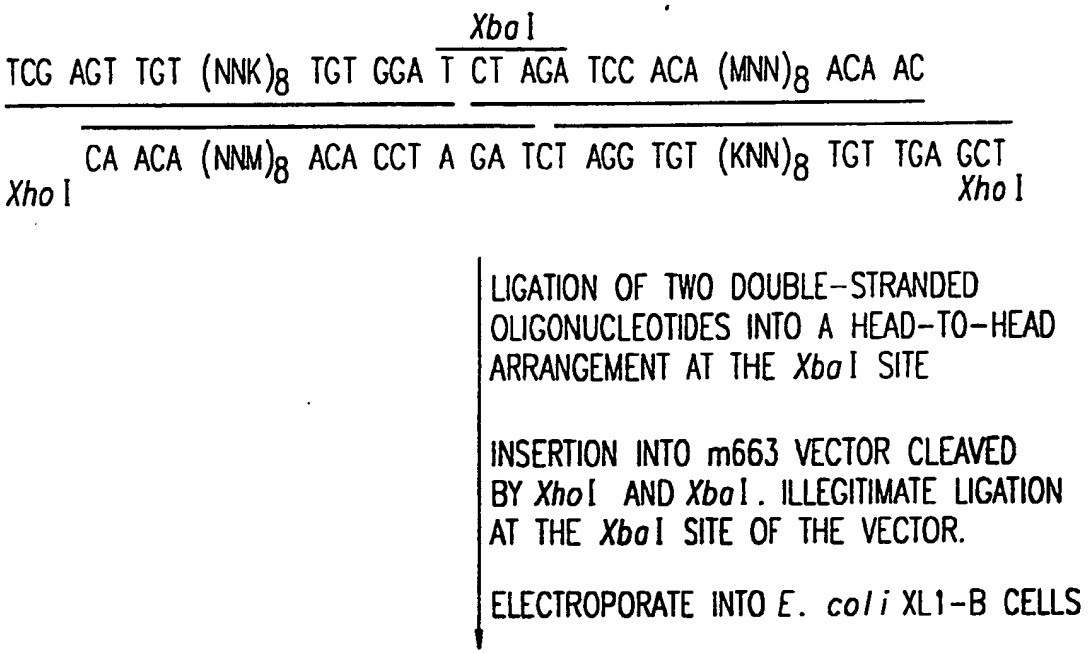

Gly
tt.ttg.tcg.acN.(NNB)10.Ngc.ggt.g
cg.cca.cNV.(NNV)10.tga.tca.ttt.t
N= A, G, T,. C
B= G, T, C
V= G, A, C
FILL IN WITH Taq DNA POLYMERASE
SalI
tt.ttg.tcg.acN.(NNB)10.Ngc.ggt.g
cg.cca.cNV.(NNV)10.tga.tca.ttt.t
SpeI
CLEAVE WITH SalI+SpeI
tcg.acN.(NNB)10.Ngc.ggt.g
cg.cca.cNV.(NNV)10.tga.tc
LIGATE WITH XhoI+XbaI -CLEAVED M13 m663 VECTOR
ELECTROPORATE INTO E. coli DH5αF'
TSAR-1.2 LIBRARY
OF pIII-RANDOM SEQUENCE FUSION PROTEINS
...S H S S (S/T) X10Ø G ∂ X10 S R P A R T ...
SIGNAL PEPTIDASE CLEAVAGE SITE
Ø = S, R, G, C, OR W
∂ = V, A, D, E, OR G

FIG.1



RESULTANT AMINO ACID SEQUENCE EXPRESSED AT THE AMINO TERMINUS OF pIII

FIG.2



RESULTANT AMINO ACID SEQUENCE EXPRESSED AT THE AMINO TERMINUS OF PIII

FIG.3

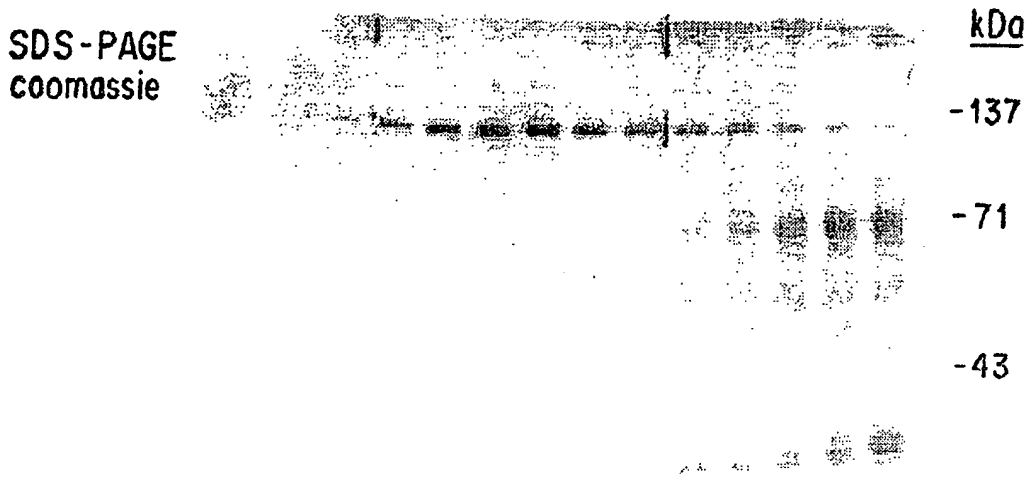


FIG. 4A

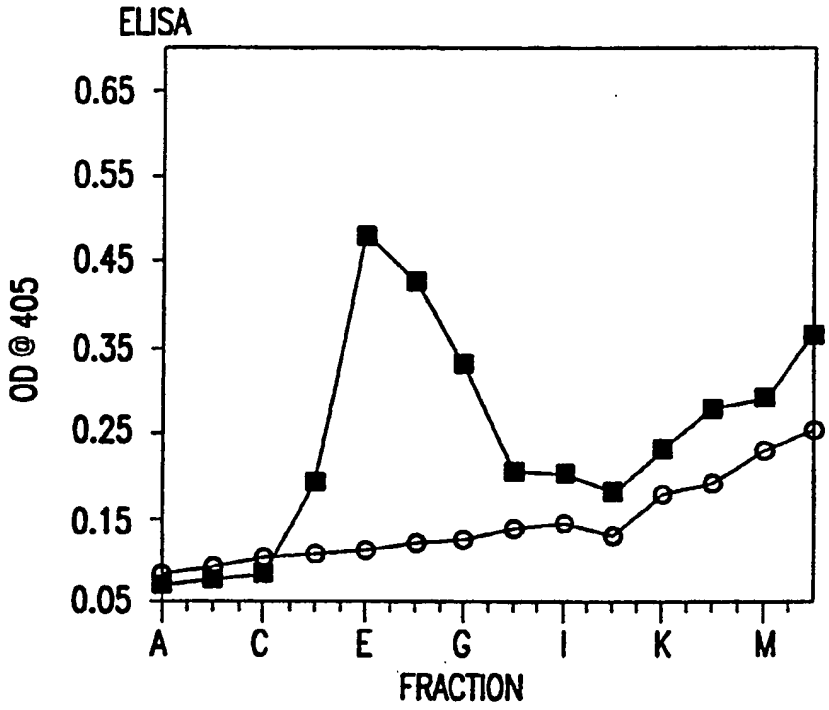


FIG.4B

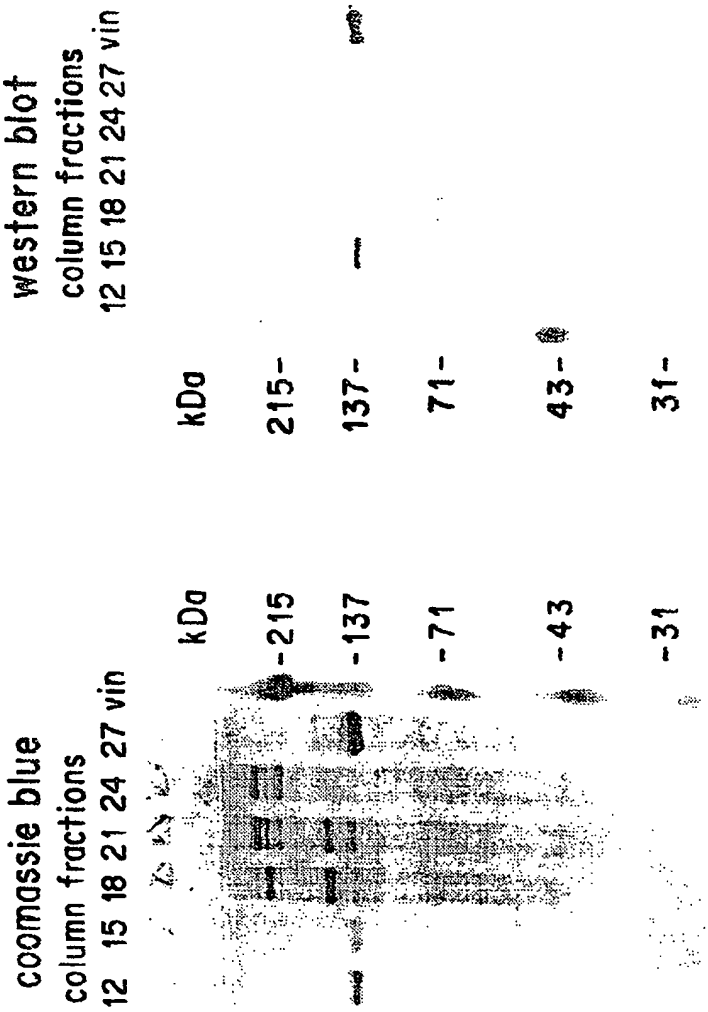

coomassie blue
column fractions
12 15 18 21 24 27 vin
kDa
-215
-137
-71
-43
-31

FIG. 5A

western blot
column fractions
12 15 18 21 24 27 vin
kDa
215-
137-
71-
43-
31-
pVin.12-1

FIG. 5B

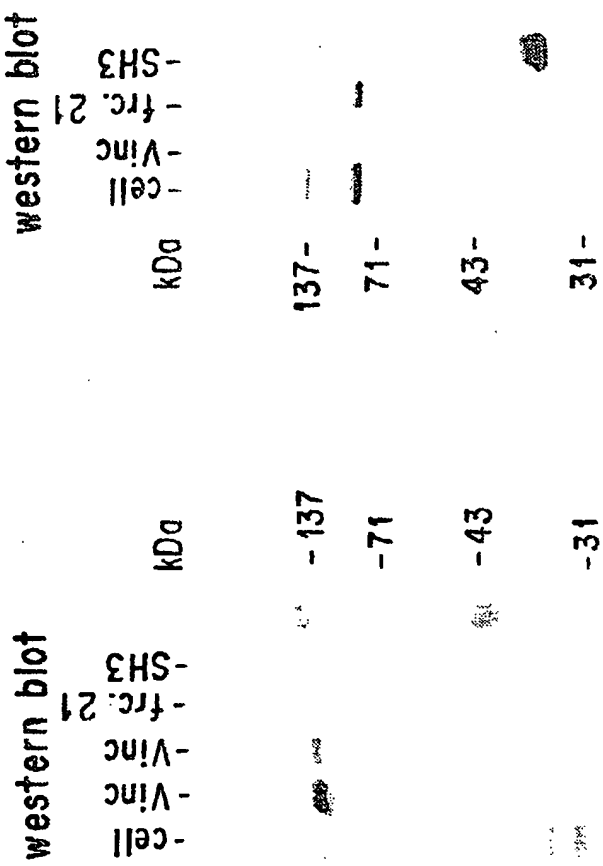

western blot
-cell
-Vinc
-frc. 21
-SH3
kDa
137-
71-
43-
31-
pA9

FIG. 6B

western blot
-cell
-Vinc
-Vinc
-frc. 21
-SH3
kDa
-137
-71
-43
-31
pVin.12-1

FIG. 6A

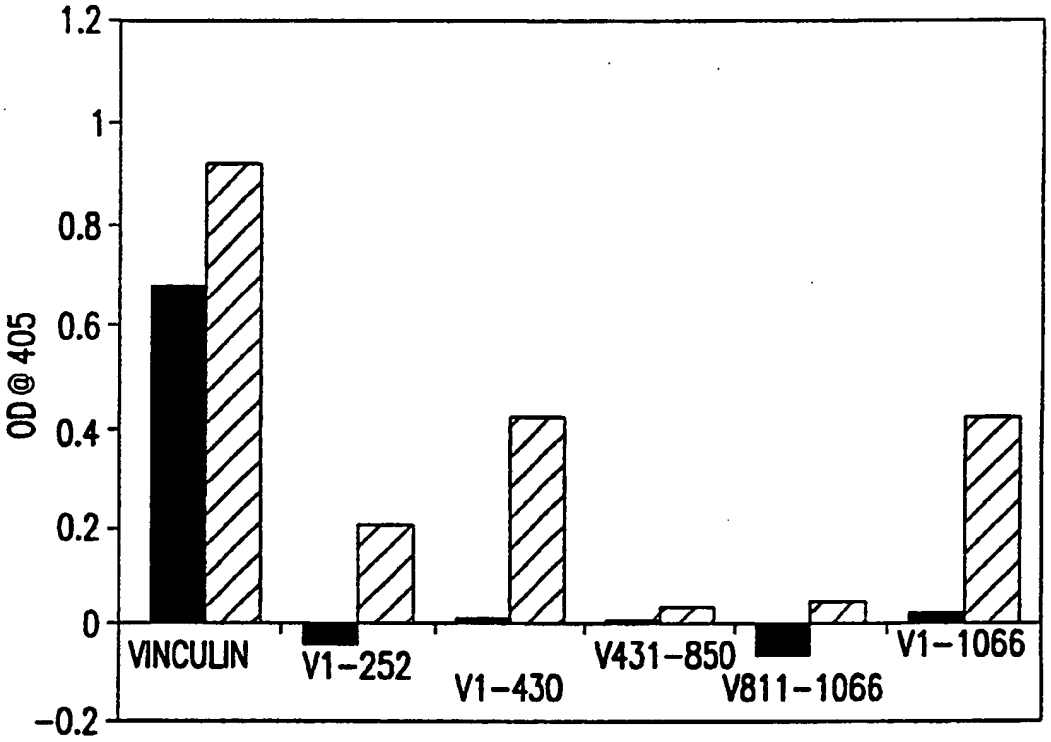

1.2
1
0.8
0.6
0.4
0.2
0
-0.2
OD @ 405
VINCULIN
V1-252
V1-430
V431-850
V811-1066
V1-1066

FIG.7

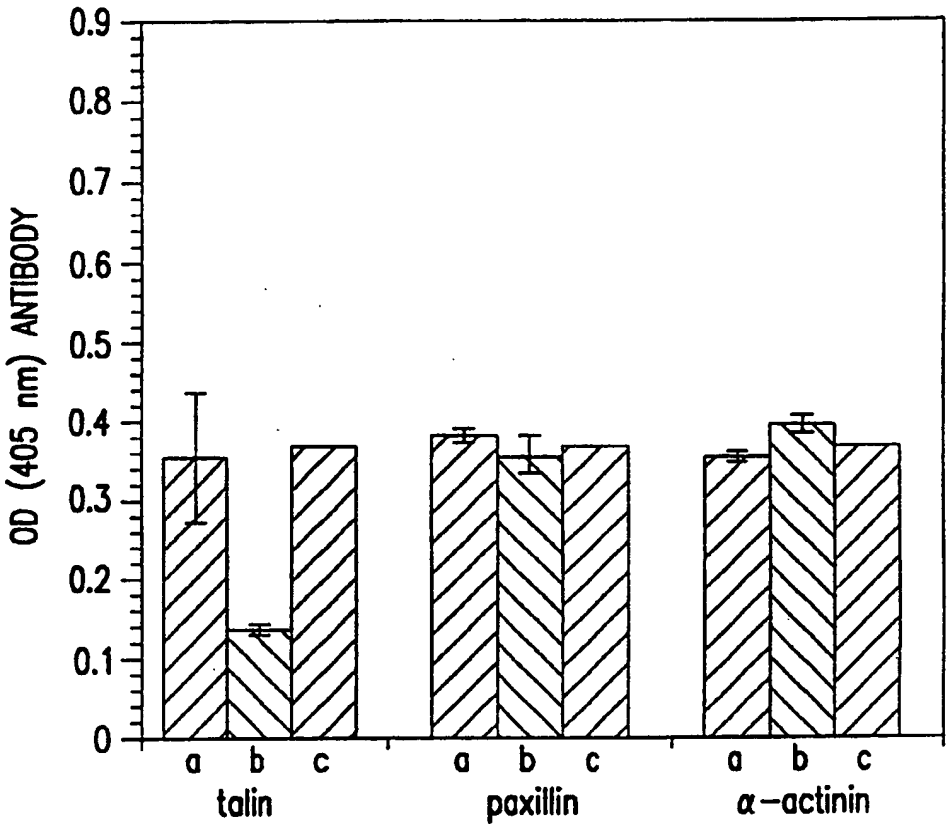

OD (405 nm) ANTIBODY
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a b c
talin
a b c
paxillin
a b c
α-actinin

FIG.8

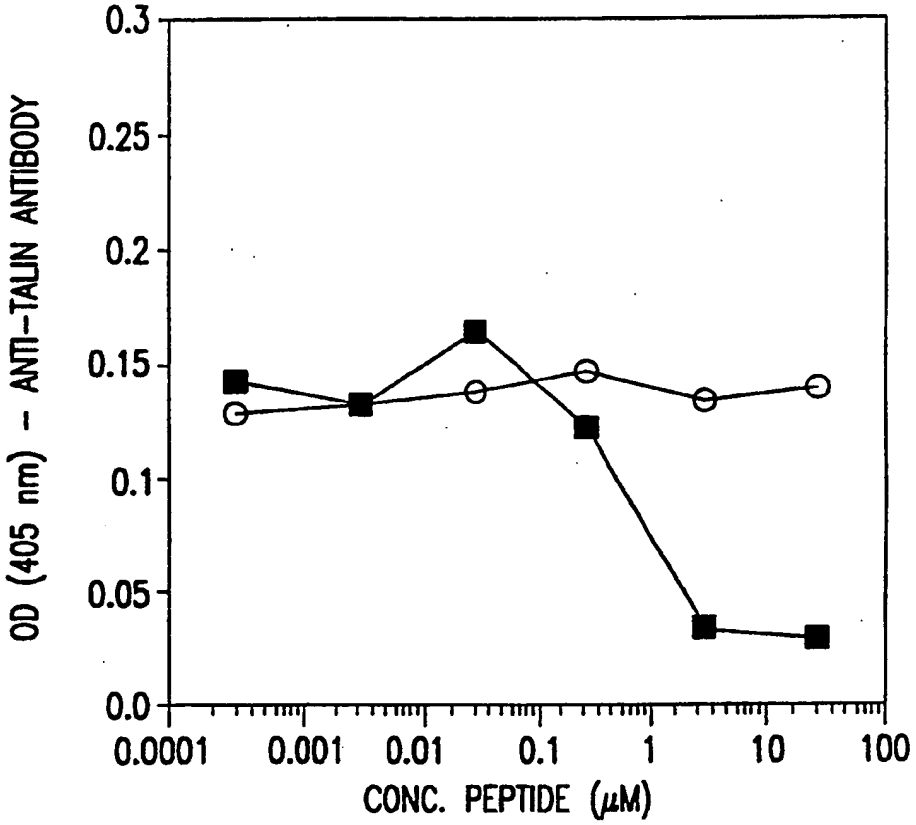

OD (405 nm) - ANTI-TALIN ANTIBODY
0.3
0.25
0.2
0.15
0.1
0.05
0.0
0.0001
0.001
0.01
0.1
1
10
100
CONC. PEPTIDE (μM)

FIG.9

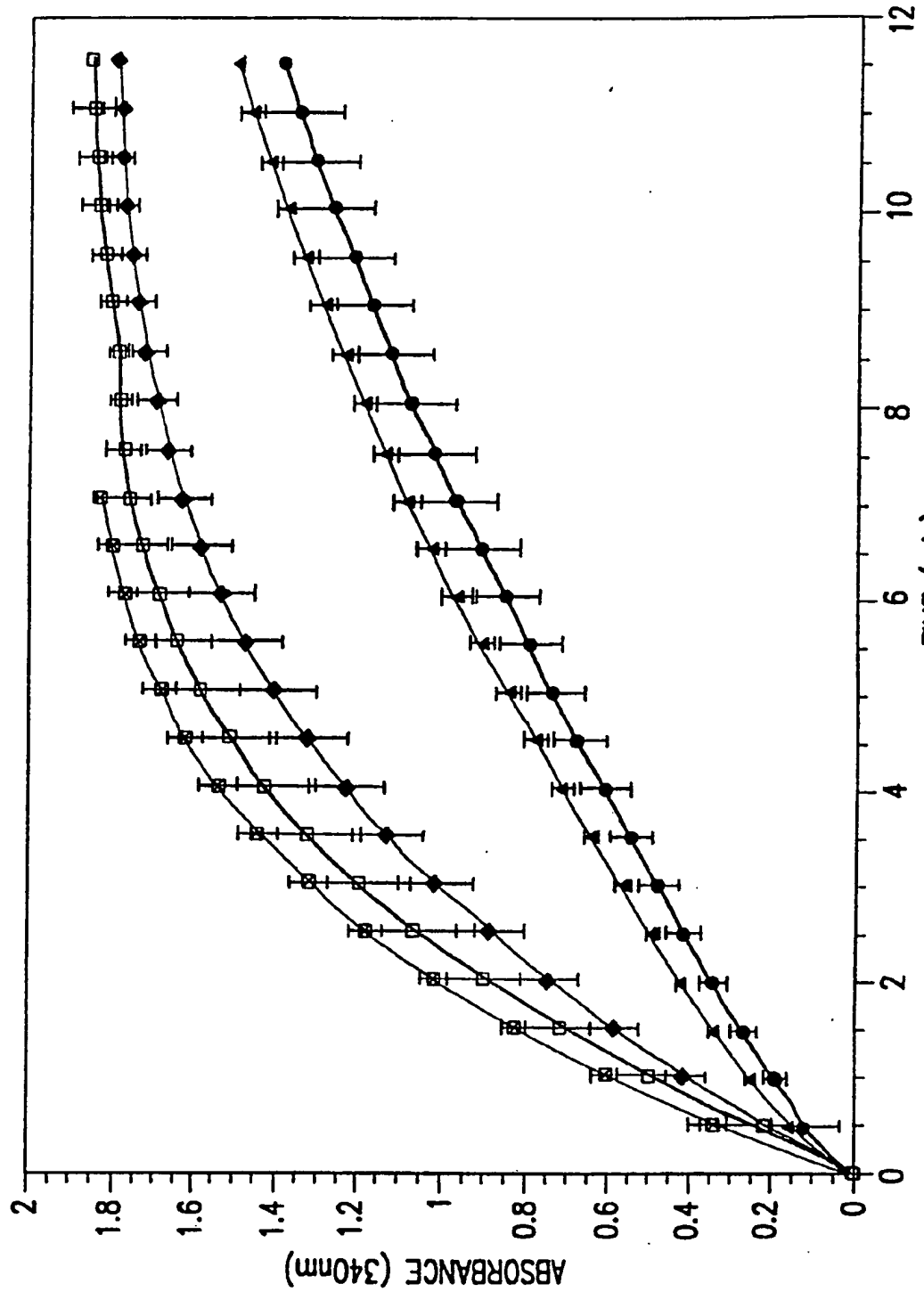

ABSORBANCE (340nm)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
TIME (min)

FIG.10

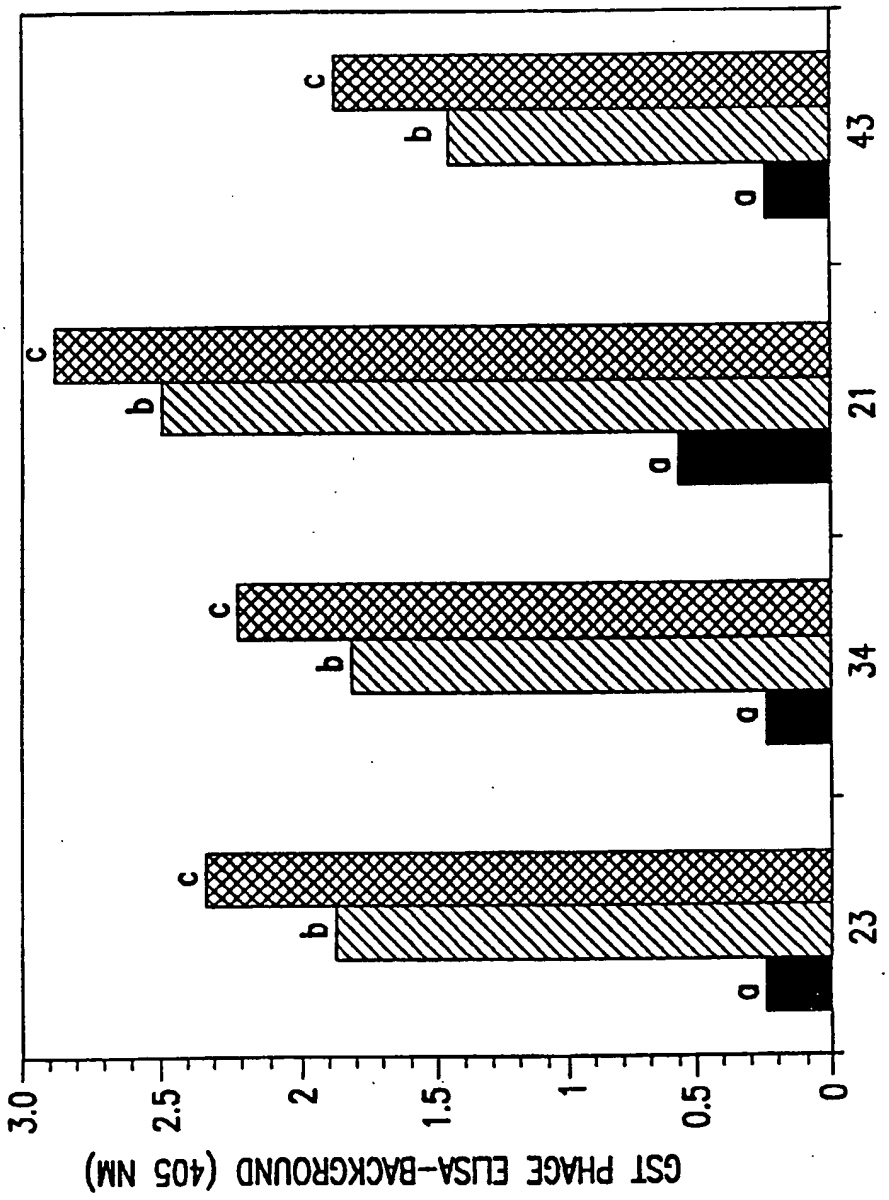

GST PHAGE ELISA-BACKGROUND (405 NM)
3.0
2.5
2
1.5
1
0.5
0
PHAGE
43
21
34
23
a
b
c

FIG.11

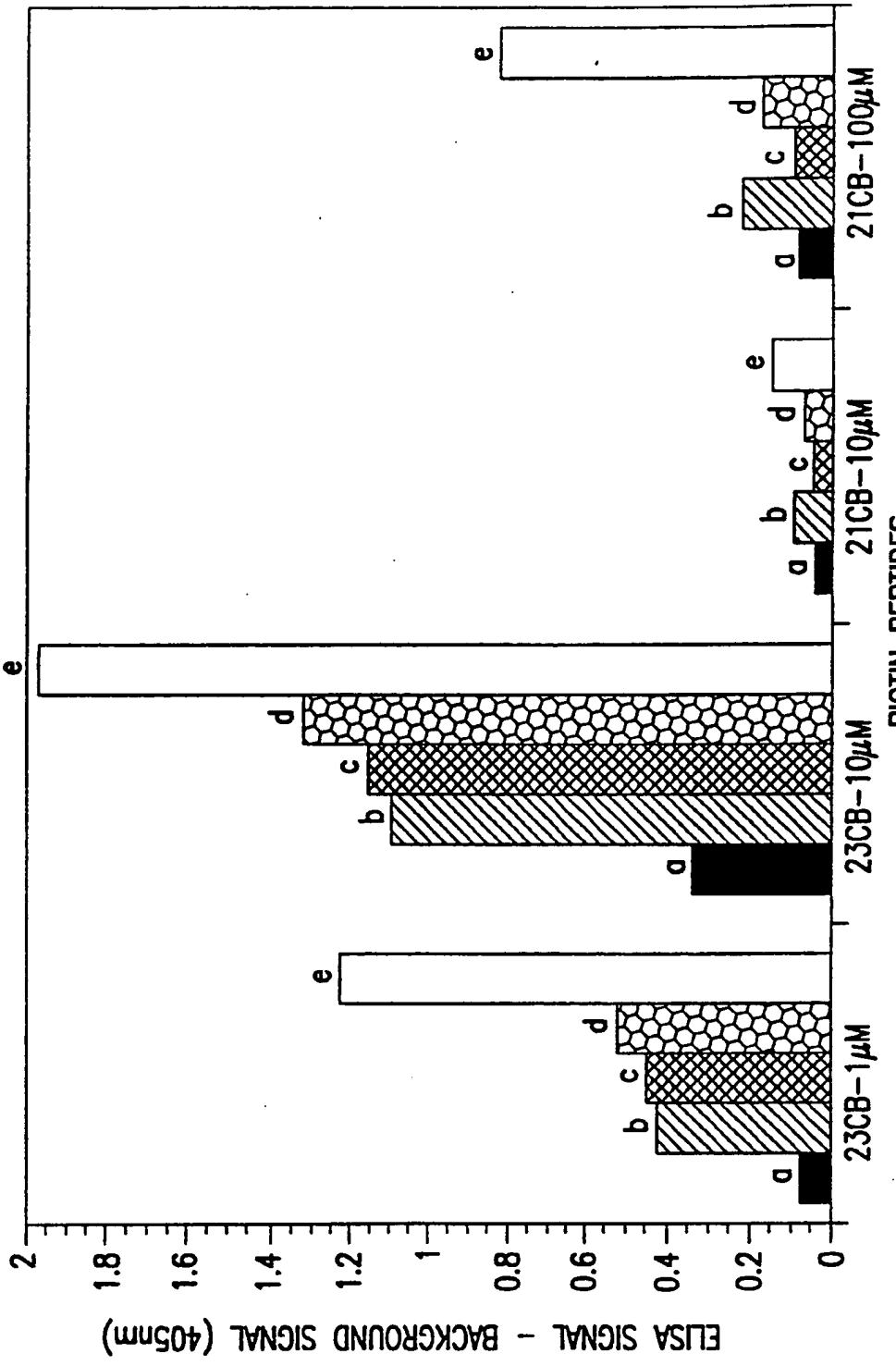

ELISA SIGNAL - BACKGROUND SIGNAL (405nm)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
23CB-1μM
23CB-10μM
21CB-10μM
21CB-100μM
BIOTIN PEPTIDES
a
b
c
d
e

FIG.12

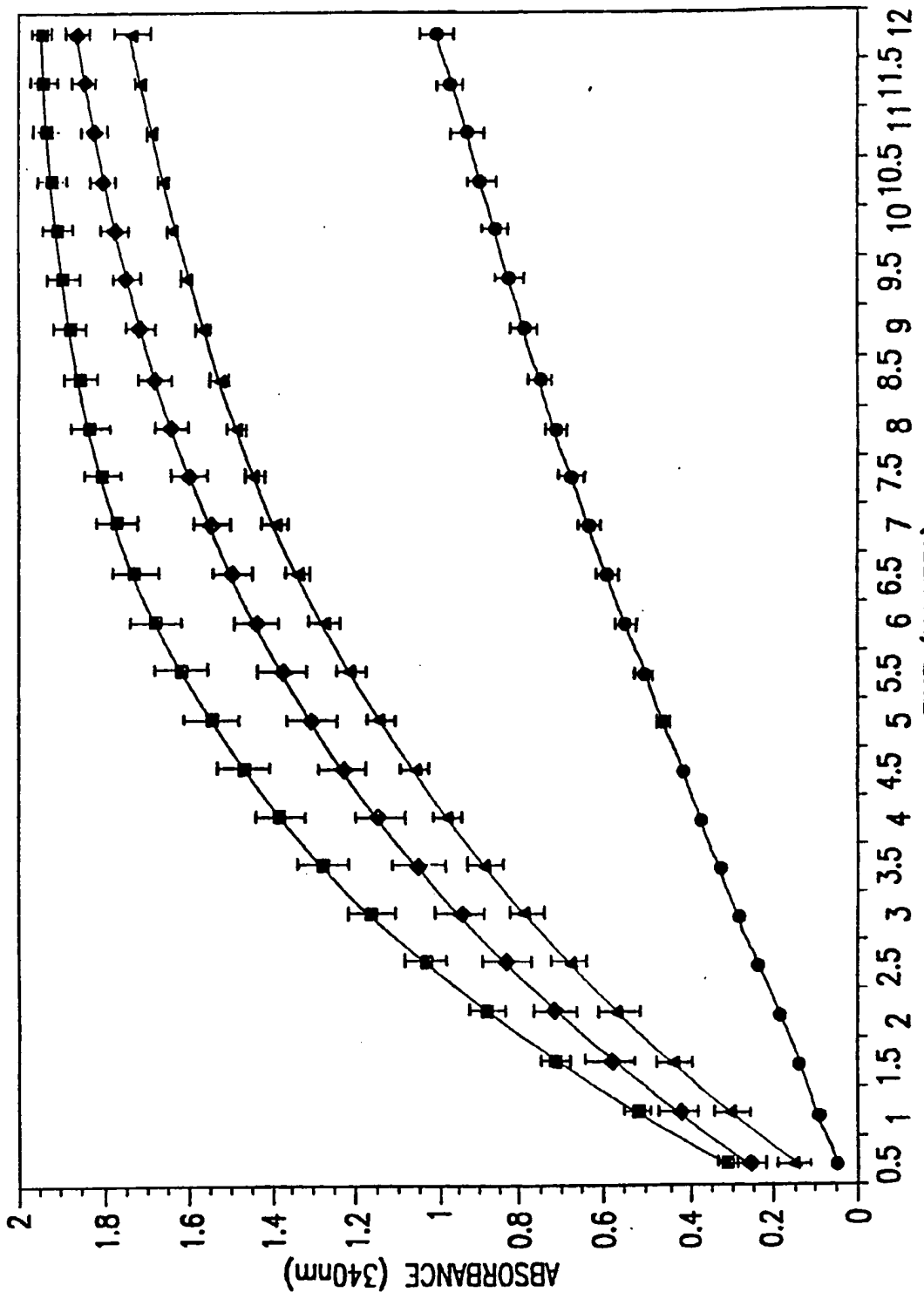

ABSORBANCE (340nm)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
TIME (MINUTES)
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8
8.5
9
9.5
10
10.5
11
11.5
12

FIG. 13

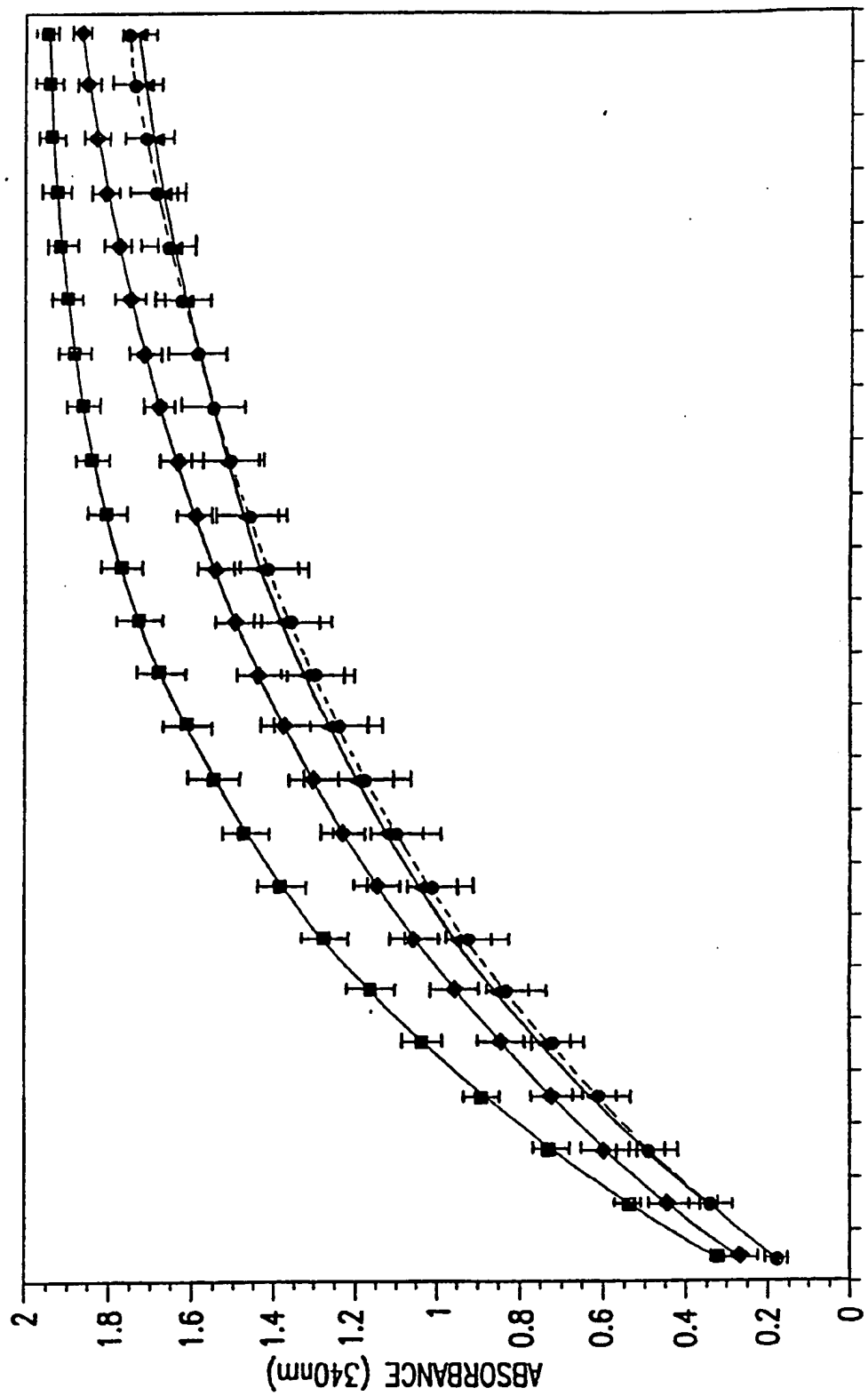

ABSORBANCE (340nm)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8
8.5
9
9.5
10
10.5
11
11.5
12
TIME (MINUTES)

FIG. 14

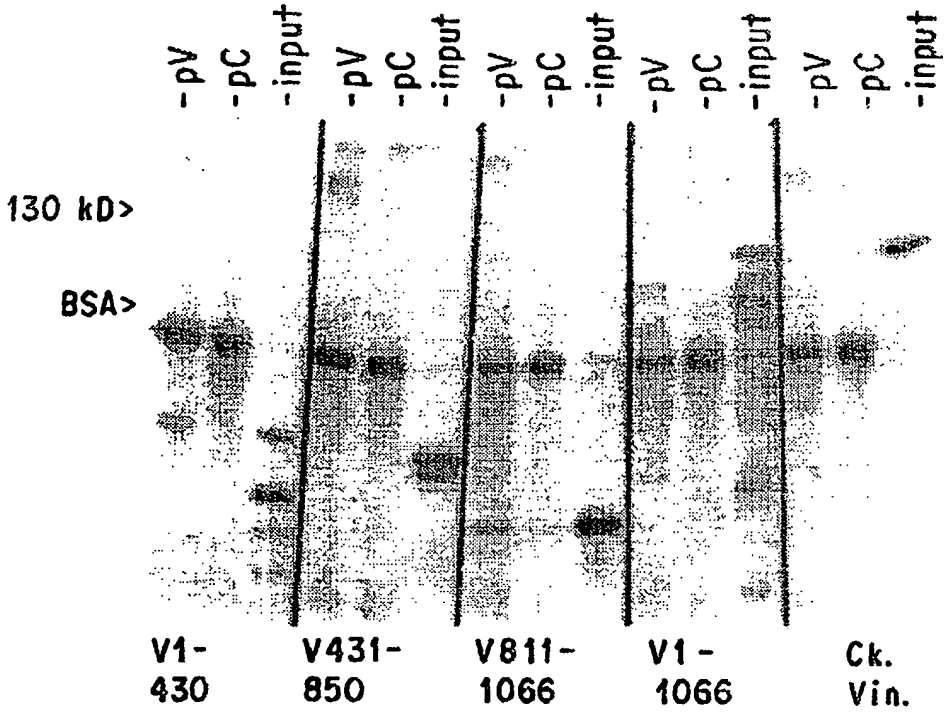

-pV
-pC
-input
-pV
-pC
-input
-pV
-pC
-input
-pV
-pC
-input
-pV
-pC
-input
130 kD>
BSA>
V1-430
V431-850
V811-1066
V1-1066
Ck. Vin.

FIG. 15

INTERNATIONAL SEARCH REPORT

Inte...ational application No.
PCT/US95/01286

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 14/00; C12N 15/09, 15/62

US CL :435/69.7, 172.3; 530/300

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.2, 69.1, 69.7, 172.3, 252.3, 320.1; 436/501; 530/300, 350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN MEDLINE
search terms: random?, librar?, peptide#

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Science, Vol. 89, Number 5, issued 01 March 1992, G. C. Cull et al, "Screening for receptor ligands using large libraries of peptides linked to the C terminus of the lac repressor", pages 1865-1869, see entire document.	1-56
Y	NATURE, Vol. 354, issued 07 November 1991, R. A. Houghten et al, "Generation and use of synthetic peptide combinatorial libraries for basic research and drug discovery", pages 84-86, see entire document.	1-56
Y	NATURE, Vol. 354, issued 07 November 1991, K. S. Lam et al, "A new type of synthetic peptide library for identifying ligand-binding activity", pages 82-84, see entire document.	1-56

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 28 APRIL 1995	Date of mailing of the international search report 03MAY1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JOHN D. ULM Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/01286

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCIENCE, Vol. 249, issued 27 July 1990, J. J. Devlin et al, "Random Peptide Libraries: A Source of Specific Protein Binding Molecules", pages 404-406, see entire document.	1-56
Y	SCIENCE, Vol. 249, issued 27 July 1990, J. K. Scott et al, "Searching for Peptide Ligands with an Epitope Library", pages 386-390, see entire document.	1-56
Y	Proceedings of the National Academy of Science, Vol. 87, issued August 1990, S. E. Cwirla et al, " Peptides on Phage: A vast library of peptides for identifying ligands", pages 6378-6382, see entire document.	1-56